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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Homeobox genes represent a class of transcription factors important in embryogenesis, organogenesis, cell growth and differentiation, and cell migration. However, there is little known about their role in regulating endothelial cell (EC) phenotype in response to proangiogenic factors secreted by breast cancer, although at least two homeobox genes have been implicated in inducing the angiogenic phenotype in ECs. We are therefore testing the homeobox gene *Gax* regulates breast cancer-induced angiogenesis through its ability to regulate the expression of downstream target genes in ECs. Using an *in vitro* tube formation assay, we have found that *Gax* expression inhibits *in vitro* angiogenesis. Moreover, by real time quantitative reverse transcriptase PCR, we have found that *Gax* expression is downregulated by proangiogenic factors and, by cDNA microarray analysis, that *Gax* downregulates pro-angiogenic adhesion molecules in ECs and upregulates the cyclin-dependent kinase inhibitor $p^{19INK4D}$. In addition, we have observed that *Gax* expression downregulates NF-κB-dependent gene expression in ECs and inhibits the binding of NF-κB to its consensus sequence. These observations will allow us to study the mechanism of *Gax*-mediated activation or repression of their expression to be studied and will form the basis for future studies that will examine in more detail the mechanism by which *Gax* activates downstream target genes and the detailed signaling pathways involved in this activation. Given the profound effect *Gax* has on endothelial cell activation, it is likely that these studies will identify new molecular targets for the antiangiogenic therapy of breast cancer. Ultimately, these same techniques will be applied to other homeobox genes implicated in regulating EC phenotype during breast cancer-induced angiogenesis.

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INTRODUCTION

Homeobox genes represent a class of transcription factors important in embryogenesis, organogenesis, cell growth and differentiation, and cell migration (1-6). However, there is little known about their role in regulating endothelial cell (EC) phenotype in response to pro- and antiangiogenic factors secreted by breast cancer cells and the surrounding normal stroma. When we originally submitted our proposal, only two homeobox genes, *HOXD3* and *HOXB3*, had been implicated in regulating tumor-induced angiogenesis (2, 7, 8). Of these, *HOXD3* expression in vascular endothelial cells has subsequently been correlated with angiogenesis in DCIS and breast cancer (9). Since then, two more (*HOXD10* and *Hex*) have been implicated by others (10-13). In addition, both before this project and as part of this project, we have developed more evidence implicating another (*Gax*) (14). Because, of the handful of homeobox genes implicated in regulating angiogenesis, only *Gax* shows a strong restriction in its expression to cardiovascular tissues in the adult (15, 16), we originally proposed to test the hypothesis that *Gax* (15-32) regulates breast cancer-induced angiogenesis through its ability to regulate the expression of specific downstream target genes in vascular endothelial cells (ECs) We based this hypothesis on our preliminary data showing that *Gax* is expressed in vascular ECs (20) and inhibits EC proliferation *in vitro* (20). Using a quantitative real-time PCR assay and *in situ* hybridization, we proposed to examine the effect of breast cancer-secreted proangiogenic peptides and and antiangiogenic therapies on *Gax* expression *in vitro* and in *in vivo* models of breast cancer angiogenesis. Next, using an adenovirus expressing *Gax* (33), we proposed to drive *Gax* expression in ECs in order to determine the effect of *Gax* expression on breast cancer angiogenesis, both *in vitro* and in *in vivo* models. Finally, because few downstream targets of *Gax* have been identified (26, 31, 33), we proposed to evaluate changes in global gene expression in ECs that result from *Gax* expression in order to identify and evaluate likely downstream targets of *Gax*. Our results will form the basis for future studies that will examine in more detail the mechanism by which *Gax* activates downstream target genes and the detailed signaling pathways involved in this activation. Given the profound effect *Gax* has on endothelial cell activation, it is likely that these studies will identify new molecular targets for the antiangiogenic therapy of breast cancer. Ultimately, these same techniques will be applied to other homeobox genes implicated in regulating EC phenotype during breast cancer angiogenesis.

BODY

Background

In order to grow and metastasize, breast malignancies are critically dependent upon inducing the ingrowth of blood vessels from the host (34, 35). Numerous studies have suggested a correlation between secretion of proangiogenic molecules and increased angiogenesis and increased likelihood of lymph node metastases with poorer prognosis in breast cancer (36, 37). Inhibition of tumor-induced angiogenesis has thus emerged in the last decade as a promising new strategy for breast cancer therapy, either alone or in combination with conventional therapies (38-41). During angiogenesis, whether physiologic or tumor-induced, vascular ECs undergo distinct changes in phenotype and gene expression, including activation of proteolytic enzymes to degrade basement membrane, sprouting, proliferation, tube formation, and production of extracellular matrix (42-44). Although the EC receptors and signaling pathways activated by proangiogenic factors secreted by breast cancer cells, such as vascular endothelial growth factor

(VEGF) (45, 46) and basic fibroblast growth factor (bFGF) (45), have been extensively studied (47-49), much less is known about the molecular biology of the downstream transcription factors activated by these signaling pathways, which then activate the genes necessary for EC phenotypic changes during breast cancer-induced angiogenesis.

Homeobox genes encode transcription factors containing a common DNA-binding motif (1, 4-6, 50). Important regulators of body plan and cell fate during embryogenesis, homeobox genes also have pleiotropic roles in many cell types in the adult and can modulate cell cycle progression and arrest, cell differentiation, migration, and apoptosis (1, 3-5, 7, 14, 51, 52). As a gene family, they are thus excellent candidates to be involved in the final transcriptional control of genes responsible for the changes in EC phenotype induced by breast cancer-secreted proangiogenic factors. Until recently, little was known about how homeobox genes might influence angiogenesis. There is now evidence for their involvement in phenotypic changes ECs undergo during angiogenesis (7, 8, 12-14). For instance, one homeobox gene, *HOXD3*, induces the expression of $\alpha_V\beta_3$, an integrin important in angiogenesis (53), resulting in the conversion of ECs to an angiogenic phenotype both *in vitro* and *in vivo* (7); impaired *HOXD3* expression is associated with impaired angiogenesis in a mouse model (52) and increased *HOXD3* expression is observed in the vasculature of breast cancer and DCIS as compared to the vasculature of the surrounding normal breast (9). Similarly, overexpression of the homeobox gene *HOXB3* results in an increase in capillary vascular density and angiogenesis (8). Taken together, these data suggest significant roles for specific homeobox genes in responding to extracellular signals and activating downstream genes to induce phenotypic changes associated with breast cancer-induced angiogenesis. Since the submission of our original proposal, two additional homeobox genes have been implicated in the regulation of EC phenotype during angiogenesis. In contrast to *HOXB3* and *HOXD3*, another HOX cluster gene, *HOXD10*, inhibits EC conversion to the angiogenic phenotype (12). *HOXD10* expression is higher in quiescent vascular endothelium in the stroma than in breast cancer-associated vascular endothelium (12). In addition, sustained expression of *HOXD10* inhibits EC migration and blocks bFGF- and VEGF-induced angiogenesis in the chick chorioallantoic membrane assay *in vivo*. Consistent with these observations, human ECs overexpressing *HOXD10* fail to form new blood vessels when embedded in Matrigel-containing sponges (12) in nude mice. Similarly, *Hex* overexpression in human umbilical vein endothelial cells (HUVECs) inhibits angiogenesis and blocks VEGF receptor signaling (11, 13). Given that previous data showing high levels of *Hex* expression in proliferating vasculature had suggested that *Hex* would be more likely to induce EC proliferation and angiogenesis (54, 55), this subsequent observation that *Hex* inhibits VEGF signaling and angiogenesis suggests a more complex role for this gene in postnatal angiogenesis than previously understood.

The cardiovascular-specific homeobox gene *Gax* appears more likely to function as a negative regulator of breast cancer-induced angiogenesis in ECs, like *HOXD10* or *Hex*. After isolating it from a rat aorta cDNA library (15, 56), we and others have shown that *Gax* has profound effects on cardiovascular tissues (19, 22, 23, 25, 26, 31, 33). In vascular smooth muscle cells (VSMCs) *Gax* expression is downregulated by mitogenic signals and upregulated by growth arrest signals (15, 32). Consistent with this observation, *Gax* induces G_1 cell cycle arrest (33) and can induce apoptosis in VSMCs under stress (25). Also, *Gax* overexpression inhibits VSMC migration, downregulating the expression of integrins, $\alpha_V\beta_3$ and $\alpha_V\beta_5$, both of which are associated with the activated ("synthetic") state in VSMCs and the angiogenic phenotype in ECs

(31, 53). *In vivo*, *Gax* expression in arteries inhibits proliferative restenosis of the arterial lumen after injury (22, 23, 26, 33). We now have evidence that *Gax* mRNA is also expressed in ECs (14). Understanding the actions of *Gax* on downstream target genes, as well as signals that activate or repress *Gax* expression, could thus lead to a better understanding of the mechanisms of breast cancer-induced angiogenesis and the identification of new molecular targets for the antiangiogenic therapy of breast cancer.

Little is known about specific transcription factors involved in the control of endothelial cell phenotype during breast cancer-induced angiogenesis, especially transcription factors that inhibit angiogenesis. *Gax* is a novel homeobox transcription factor (15) whose antiproliferative (31), pro-apoptotic (25), and antimigratory (31) activities were originally characterized by us and others in vascular smooth muscle. Although other homeobox genes have been implicated in promoting or inhibiting EC conversion to the angiogenic phenotype (7, 8, 12-14), *Gax* is the only homeobox transcription factor described thus far whose expression is largely restricted to the cardiovascular system in the adult (15, 16). It is thus a new and unique candidate transcription factor for regulating EC conversion to the angiogenic phenotype in response to proangiogenic factors secreted by breast tumors. The hypothesis that *Gax* inhibits the phenotypic changes in ECs that occur when they are stimulated by the proangiogenic factors secreted by breast cancer cells represents an innovative approach to the study of the transcriptional control of EC phenotypic changes during angiogenesis, as does our use of *Gax* as a molecular tool to study the mechanisms of breast cancer-induced angiogenesis. More importantly, identification of downstream targets of *Gax* could identify previously unsuspected molecular targets for the antiangiogenic therapy of breast cancer and other tumors, leading to new lines of investigation into breast cancer-induced angiogenesis and new therapies based on these observations. Thus, the studies we have proposed and undertaken with support from the Department of Defense have attempted to use *Gax* as a molecular tool to: (1) enhance our understanding of the mechanisms by breast cancer stimulates endothelial cells to become angiogenic; and (2) provide the basis for the design of antiangiogenic therapies of breast cancer targeting *Gax* or its downstream targets.

Overview of progress over the last year

Since this project began, we have made considerable progress in meeting the milestones originally proposed in our original Statement of Work. Most of the tasks originally proposed for Year One are on schedule, and we are ahead of schedule on some tasks originally scheduled to be completed in year two or three. However, we do note that we are behind schedule on certain tasks, particularly Tasks 1a and 1b. Of particular interest, in analyzing our early cDNA microarray experiments, we made an unexpected observation. We noted that *Gax* expression downregulates NF-κB-dependent gene expression in ECs. We then confirmed that Gax expression

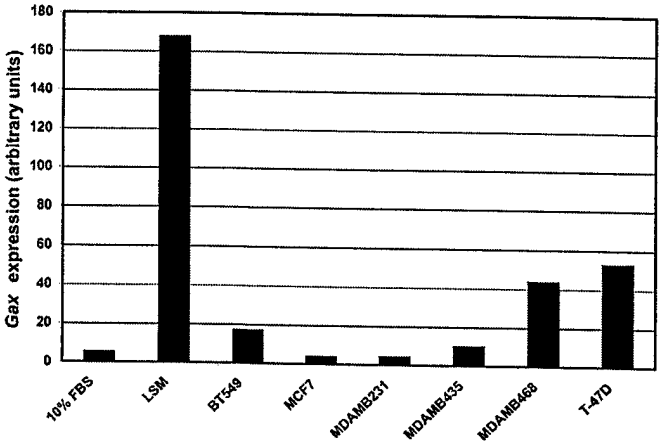

Figure 1. Downregulation of *Gax* expression in endothelial cells by conditioned medium from tumor cell lines. Quiescent HUVECs were treated with either low serum medium (LSM), 10% FBS, or 10% conditioned medium from the indicated breast cancer cell lines. Cells were harvested 4 hours after stimulation, total RNA harvested and real time quantitative RT-PCR performed. *Gax* message level was normalized to GAPDH. Units are arbitrary.

blocks NF-κB binding to its consensus sequence in electrophoretic mobility shift assays (EMSAs). This observation has suggested an entirely new area of research into the mechanism by which Gax expression inhibits angiogenesis, as there is now considerable evidence that NF-κB activity is proangiogenic in ECs. Consequently, early in year two, we will evaluate our progress and determine whether we need to submit formal changes to our Statement of Work in order to pursue this new observation and to determine if *Gax* may inhibit breast cancer-induced angiogenesis.

Detailed progress report by tasks in the original Statement of Work

Task 1:Measure differences in Gax expression between angiogenic blood vessels and normal blood vessels in vivo (months 1 to 24).

a. Measure levels of proangiogenic factors in six breast cancer tumor cell lines (months 1-3)

Status: In progress. Because of the potentially important finding that *Gax* appears to inhibit NF-κB signaling in vascular ECs (see Task 4), this fall we decided to defer the bulk of these experiments until Year Two. We will finish these experiments during Year Two.

b. Measure breast cancer cell line-induced angiogenesis in vivo *using the Matrigel plug assay and breast cancer cell line-conditioned media, and measure* Gax *expression in endothelial cells* in vivo. *(months 1-12).*

Status: In progress. Because endogenous *Gax* message is usually expressed at relatively low levels in VSMCs and ECs (14, 15), we developed a quantitative real time PCR assay using *Gax*-specific primers and a TaqMan probe (57). Using this assay, we completed the *in vitro* experiments and found that, for nearly every breast cancer cell line we have studied, serum-free media conditioned for 24 hours by breast cancer cells strongly downregulated *Gax* expression in

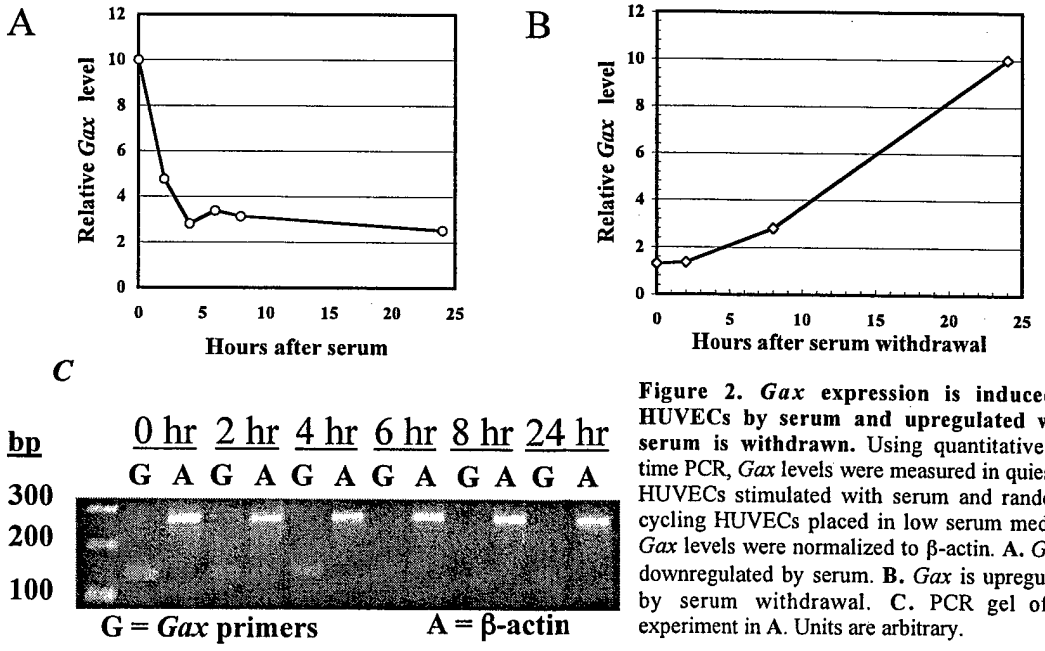

Figure 2. *Gax* expression is induced in HUVECs by serum and upregulated when serum is withdrawn. Using quantitative real time PCR, *Gax* levels were measured in quiescent HUVECs stimulated with serum and randomly cycling HUVECs placed in low serum medium. *Gax* levels were normalized to β-actin. **A.** *Gax* is downregulated by serum. **B.** *Gax* is upregulated by serum withdrawal. **C.** PCR gel of the experiment in A. Units are arbitrary.

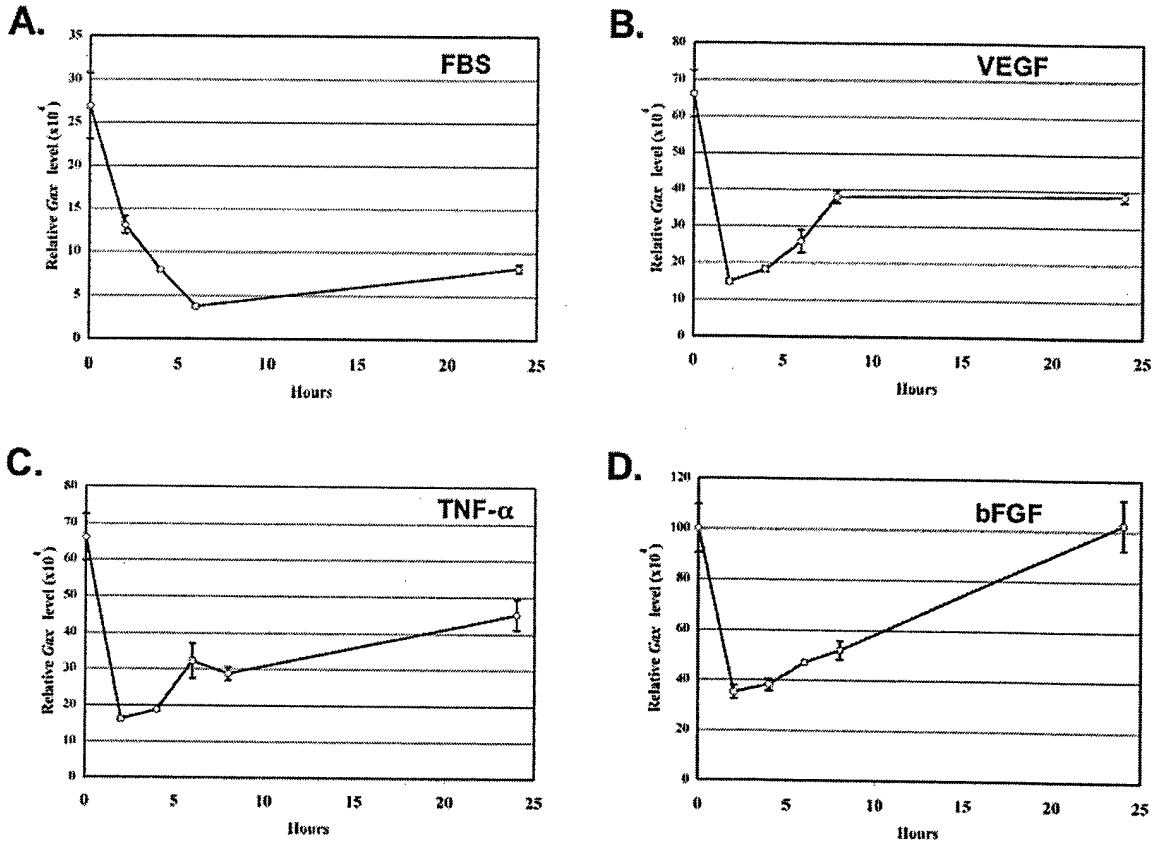

Figure 3. Time course of *Gax* downregulation by mitogens and proinflammatory factors. Quiescent HUVECs were treated with either **(A)** 10% fetal bovine serum (FBS); or 10 ng/ml of either **(B)** $VEGF_{165}$; **(C)** TNF-α; or **(D)** bFGF. At various time points, cells were harvested for extraction of total RNA, which was then subjected to quantitative real time TaqMan RT-PCR with *Gax*- and GAPDH-specific primer/probe sets. *Gax* mRNA levels were normalized to GAPDH. Units are arbitrary. E. Cells were treated identically to **(C)**, except that after six hours cells were harvested for protein extraction and then subjected to Western blot with Gax-specific polyclonal antibody.

ECs within four hours. Two cell lines, MCF7 and MDA-MB231, were as potent as fetal bovine serum in downregulating *Gax* (Figure 1).

Next, to begin identifying which factors secreted by breast cancer cells are likely to be the ones that result in downregulation of *Gax* expression, we have followed up these observations by examining the effect of VEGF, bFGF, and TNF-α on *Gax* message levels using quantitative real time PCR (Figure 2). In all cases, *Gax* was rapidly downregulated and then more slowly returned to baseline after stimulation with proangiogenic factors. First, we studied the time course of *Gax* downregulation. HUVECs made quiescent by incubation for 24 hrs in 0.1% FBS were stimulated with 10% FBS plus 5 ng/ml VEGF. *Gax* was rapidly downregulated by 5-fold within four hours and slowly returned to basal over 24 to 48 hours (Figure 2, A and C). Conversely, when sparsely plated randomly cycling HUVECs were placed in medium containing 0.1% serum, *Gax* was upregulated nearly 10-fold within 24 hours (Figure 2B). We then stimulated quiescent HUVECs with proangiogenic or proinflammatory factors, including bFGF,

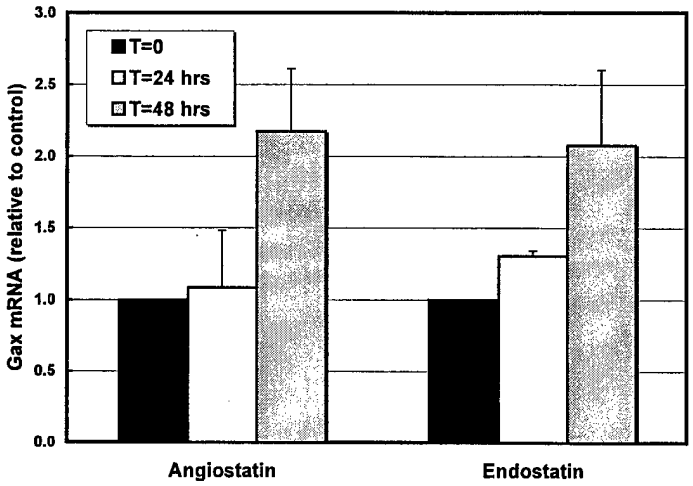

Figure 4. Upregulation of Gax by antiangiogenic peptides. Randomly cycling HUVECs were treated with either angiostatin or endostatin at 1 μg/ml. At varying time points, cells were harvested for RNA isolation, which was then subjected to reverse transcriptase quantitative real time PCR. Gax mRNA levels were normalized to GAPDH and expressed as ratios to Gax levels in control HUVECs allowed to incubate in parallel in normal medium. $p<0.01$ at 48 hrs for angiostatin and endostatin.

VEGF, and TNF-α. *Gax* was rapidly downregulated with a similar time course (Figure 3). Similar results were observed in HMEC-1 cells, an immortalized human microvascular endothelial cell line (58) that retains many characteristics of microvascular endothelial cells (data not shown). Finally, we examined whether antiangiogenic peptides that might be used, either alone or in combination (59, 60), to treat breast cancer affected *Gax* expression. Randomly cycling HUVECs were incubated for varying times with 1 μg/ml angiostatin (59) or endostatin (60). Cells were harvested for total RNA isolation and the RNA then subjected to quantitative real time PCR to measure *Gax* expression. We found that both angiostatin and endostatin upregulated *Gax* expression by two-fold over 48 hours, a time course that was slower and an upregulation that was less dramatic than that caused by serum deprivation (Figure 4). From these results, we are now preparing to test the ability of *Gax* to block angiogenesis due to breast cancer-conditioned media in the Matrigel plug assay, as we have described in Task 3 (see below).

c. *Compare immunohistochemical staining and labeling by* in situ hybridization *for* Gax *expression in breast tumor blood vessels with that of blood vessels found in normal breast for 50 invasive human breast cancer specimens (months 12-24).*

Status: In progress. We have recently begun these experiments. We began by using mouse tissues to optimize conditions for our antibody and have recently begun to do *in situ* hybridization using a probe for *Gax* that does not include its homeodomain or CAX repeat (15, 21). These are presently at too early a stage to report the results and will be described in next year's report.

Task 2: Determine the effects of Gax overexpression in endothelial cells in vitro (months 1-24).

a. *Determine effect of* Gax *overexpression and blockade on endothelial cell proliferation and expression of cell cycle regulatory genes. (months 1-12).*

Status: In progress. Using cDNA microarray experiments, we have identified several cyclin dependent kinase inhibitors that are upregulated by Gax expression, including $p19^{INK4D}$, $p57^{Kip2}$, and $p21^{WAF1/CIP1}$ (33, 61, 62). These experiments will be described in more detail in Task 4. The upregulation of these CDK inhibitors suggests redundant mechanisms by which *Gax* can induce G_1 cell cycle arrest. We have also shown that the upregulation of p21 in ECs is due to a

p53-independent activity of Gax on the $p21^{WAF1/CIP1}$ promoter [(14), in Appendix]. What remains is (1) to verify the upregulation of $p57^{Kip2}$ and $p19^{INK4D}$ and investigate the mechanism by which *Gax* accomplishes this upregulation.

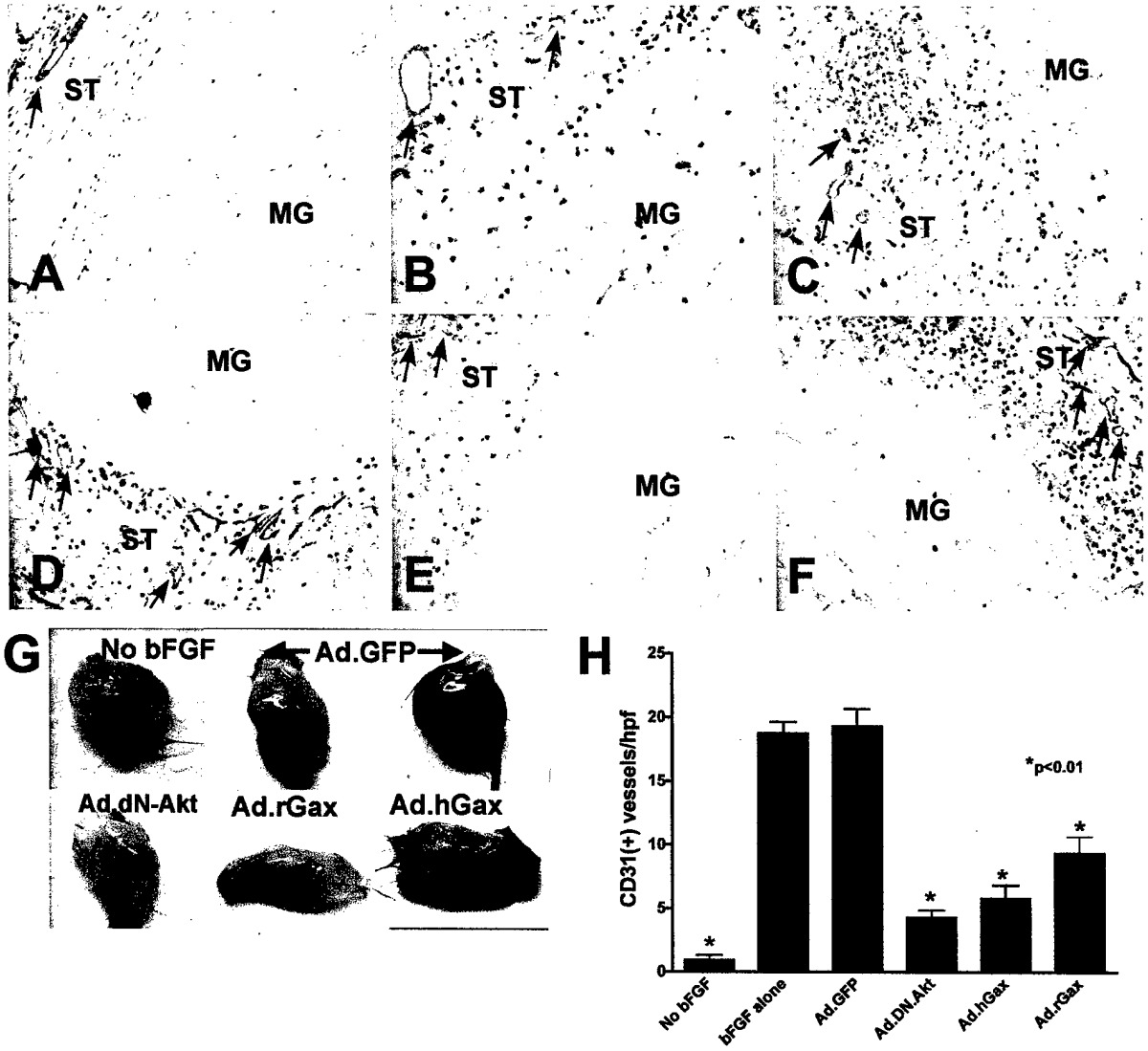

Figure 5. Effect of *Gax* expression on angiogenesis in Matrigel plugs. Matrigel plugs (500 ul each) containing 400 ng/ml bFGF and the indicated viral constructs at 10^8 pfu/plug were implanted subcutaneously in the flanks of C57BL6 mice. Plugs were harvested after 14 days incubation for immunohistochemistry using CD31 antibodies and determination of CD31-postitive cells per high powered (400x) field. Slides were photographed at 200x magnification. (**Legend:** MG = Matrigel plug; ST = stroma surrounding the plug; arrows indicate examples of CD31-positive blood vessels.) **A. No growth factor. B. bFGF alone, no virus. C. Ad.GFP.** Note the infiltration of the plug with CD31-positive vessels such that it is difficult to determine the exact edge of the plug in **B** and **C**. **D. Ad.dN.Akt. E. Ad.h*Gax*. F. Ad.r*Gax*. G. Gross photographs of selected plugs.** Note the hemorrhage into one of the Ad.GFP plugs and the lack of vessels on the capsule of the Ad.*Gax* and Ad.dN.Akt plugs. **H. Vessel counts.** Results are plotted as means ± standard error of the mean, and statistical differences determined with one-way ANOVA $p<0.0001$ for the overall, and the vessel counts were statistically significantly different from control (Ad.GFP group) for Ad.DN.Akt ($p=0.013$); Ad.h*Gax* ($p=0.008$); and Ad.r*Gax* ($p=0.028$).

b. *Determine effect of* Gax *overexpression and blockade on expression of pro-angiogenic integrins, specifically if the expression of integrins* $\alpha_V\beta_3$ *and* $\alpha_V\beta_5$ *are regulated by* Gax *expression (Months 6-18).*

Status: In progress. We have recently begun to look more closely at the effect of *Gax* expression on the expression of integrins and anticipate these experiments being completed in Year Two.

c. *Characterize* Gax*-induced endothelial cell apoptosis and the effect of* Gax *expression and blockade on the expression of genes regulating apoptosis (months 13-24).*

Status: Not done. We plan to begin these experiments in Year Two.

d. *Determine whether* Gax *expression and blockade alters the activity of two major signaling pathways implicated in endothelial cell angiogenesis (months 13-24).*

Status: In progress. We have identified three potential signaling pathways that are influenced by *Gax* expression. These pathways include NF-κB (63), Wnt (64, 65), and transforming growth factor-β (66, 67). Of these, we have verified that one of them, NF-κB, is definitely inhibited by *Gax* activity, thus completing half of this task. We will now concentrate on determining if *Gax* activity influences Wnt and transforming growth factor-β (TGF-β) signaling in ECs. See Task 4 for a more detailed discussion of how we identified these pathways from our cDNA microarray data.

Task 3: Determine the effects of Gax overexpression on angiogenesis in vivo. (Months 13-36.)

a. *Matrigel plug assays in C57BL/6 mice to determine if Ad.*Gax *inhibits* in vivo *angiogenesis and to quantify how strong the effect is (months 13-36).*

Matrigel containing proangiogenic factors, when implanted subcutaneously in mice, can stimulate the ingrowth of blood vessels into the Matrigel plug from the surrounding tissue, and this neovascularization can be estimated by counting CD31-positive cells and/or by measuring hemoglobin

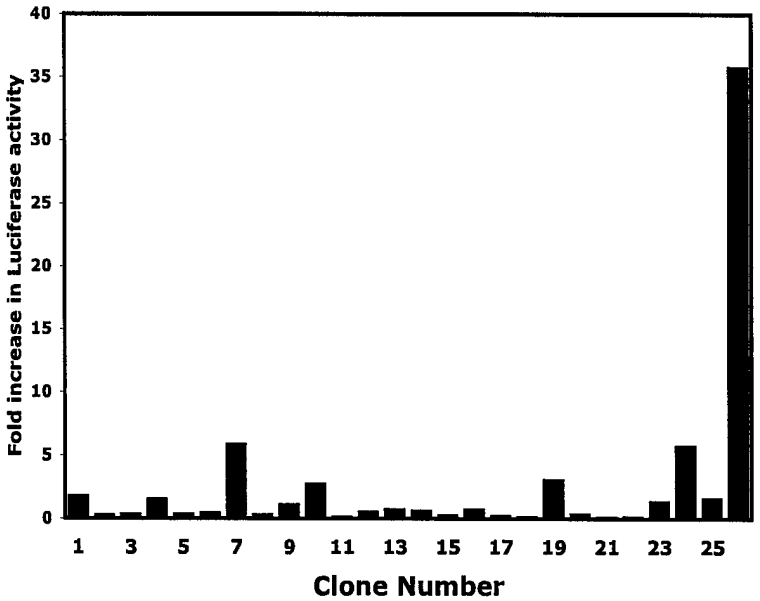

Figure 6. Tetracycline-inducible HMEC-1 constructs. HMEC-1 cells were transduced with pTet-On, which introduces the rTA element. Cells were selected with Hygromycin B, and then Hygromycin B-resistant colonies selected and expanded. Cells from individual colonies were then transduced with pTRE-Luc, a plasmid in which Luciferase expression is driven by the Tet response element, which is active in the presence of tetracycline or doxycycline and silent otherwise. Luciferase expression was determined in the presence and absence of doxycycline. Clone #26 showed the most induction with doxycyline.

concentrations in the plug (68). Moreover, adenoviral vectors diluted in Matrigel implanted as subcutaneous plugs can serve as reservoirs to transduce ECs invading the plug and drive expression of exogenous genes (69, 70), producing effects on *in vivo* angiogenesis even when the gene transduced is a transcription factor (71). As originally proposed, we have taken advantage of this observation to test whether exogenously driven *Gax* expression can inhibit angiogenesis *in vivo*, using methodology previously described. Matrigel plugs containing bFGF and either Ad.GFP, Ad.*hGax*, or Ad.*rGax* (see manuscript in Appendix) were injected subcutaneously in C57BL/6 mice (N=8 per experimental group). As a positive control for angiogenesis inhibition by a viral vector, we utilized an adenoviral construct expressing a dominant negative form of Akt (Ad.DN-Akt) (69, 70). We observed that the adenoviral vectors expressing *Gax* expression inhibit the neovascularization of the plugs with a potency slightly less than that observed for the Ad.DN-Akt construct (Figure 5), and that the Ad.DN.Akt construct inhibited neovascularization with a potency similar to what has previously been reported (69, 70). The results of these experiments indicate that Gax is capable of inhibiting angiogenesis in in vivo models and will form the basis of experiments proposed in Task 4.

b. *Matrigel plug assays using tumor cells from breast cancer cell lines to determine if Ad.*Gax *inhibits* in vivo *angiogenesis and to quantify how strong the effect is (months 24-36).*

Status: Not begun yet. These experiments are to be done in Year Three.

c. *Chick chorioallantoic membrane assays to quantify* Gax *inhibition of angiogenesis (months 13-36).*

Status: Not begun yet. We plan to begin these experiments in Year Two.

Task 4: Identify potential downstream targets of Gax (months 1 through 24).

a. *Construct stably transfected endothelial cells with tetracycline-inducible* Gax *expression and verify inducible* Gax *expression (months 1 to 9).*

Status: In progress. We are somewhat behind schedule in constructing the stable transfectants. However, we have successfully generated several clones based on HMEC-1 cells, an immortalized human microvascular endothelial cell line (58), with the Tet-On system (Clontech). These cells have varying levels of doxycycline-inducible gene activity when plasmids containing the Luciferase

Table 1: Genes downregulated by *Gax* expression.

Gene	Fold decrease
CXCL1 (GRO-1/Gro-α)	238.0
Chemokine (C-C motif) ligand 20	237.6
Interleukin-8	181.3
Chemokine (C-X-C motif) ligand 3	119.9
Chemokine (C-X-C motif) ligand 2	79.6
E-selectin	62.6
Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	17.5
Vascular cell adhesion molecule-1	13.0
VEGF-C	5.3
Matrix metalloproteinase 10	4.3
Angiopoietin-1	3.9
Ephrin A1	3.0
Endothelin-1	2.9
Fibroblast growth factor-2 (basic)	2.8
Matrix metalloproteinase 14	2.8
Id3	2.7
Intercellular adhesion molecule-1	2.5
Id1	2.1
Id4	2.1
Endothelial-specific molecule-1 (ESM-1)	2.1

Table 2. Selected NF-κB-influenced genes downregulated by *Gax* expression (*Implicated in angiogenesis)

Gene	Fold decrease
CXCL1 (GRO-1/Gro-α)*	238.0
Interleukin-8*	181.3
Gro-β	97.0
E-selectin*	62.6
Granulocyte chemotactic protein-2	17.5
VCAM-1*	13.0
A20 (TNF-α-induced protein-3)	7.5
TNF-α receptor-associated protein-1 (TRAF-1)*	4.0
Tissue factor*	3.0
Intracellular adhesion molecule-1 (ICAM-1)*	2.5
Endothelin-1	2.5
Heparin-binding EGF	2.1
Endothelial-specific molecule-1 (ESM-1)*	2.1
Interferon regulatory factor-1*	1.7
B94 (TNF-α-induced protein-2)	1.7
Monocyte chemotactic protein-3	1.4

gene under control of the Tet response element (TRE). Using the Tet-On system, we have generated HMEC-1 clones with constitutive expression of rTA. When these cells are transduced with a reporter construct in which Luciferase is driven by the Tet response element (TRE), expression of reporter gene is induced by exposure to doxycycline (Figure 6). There are several candidate clones with tetracycline-inducible expression. The most promising is clone #26. We will use this clone as the basis to transfect with pTRE-Gax, the construct we have made in which expression of the *Gax* cDNA is controlled by TRE to produce tetracycline-inducible *Gax* expression. Completion of the construction of these stable transfectants is presently in progress.

b. Compare global gene expression between Gax*-expressing endothelial cells and non-*Gax*-expressing endothelial cells using cDNA microarrays (months 10 to 18).*

Status: In progress. Because we were behind schedule in producing ECs with tetracycline-inducible *Gax* expression (Task 4a), we temporarily pursued a different strategy to identify changes in global gene expression due to *Gax* while we continued work on our stable transfectants. We compared global gene expression in control HUVECs infected with Ad.GFP with that of HUVECs infected with Ad.r*Gax*. Cells were infected at an MOI=100, incubated 24 hours in normal media, then harvested for total RNA isolation. Global gene expression was compared in two separate experiments using the Affymetrix Human Genome U133A GeneChip® array set (see Methods). In general, the global changes in gene expression induced by *Gax* in this experiment were consistent with an anti-proliferative, antiangiogenic activity. There were 127 probe

Table 3: Selected genes upregulated by *Gax* expression

Gene	Fold increase
Frizzled homolog 2	30.4
Rab coupling protein	30.1
ALK3 (Bone morphogenetic protein receptor, type Ia)	29.7
Aquaporin 3	19.9
Frizzled	9.8
Pro-alpha I chain of type I collagen	6.4
Thrombomodulin	5.5
Id2	4.6
Integrin β_4 subunit	4.2
β_2-arrestin	2.6
$p19^{INK4D}$	2.5
Insulin-like growth factor binding protein-1	2.5
Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	2.1
HOXA5	2.1
Angiopoietin-2	2.0
$p21^{WAF1/CIP1}$	1.5

sets corresponding to known genes showing greater than two-fold upregulation and 115 showing greater than two-fold downregulation. Differences in gene expression between controls and *Gax*-transduced cells ranged from upregulation by approximately 30-fold to downregulation by 238-fold. This pattern was similar in ECs transduced by Ad.h*Gax*, although the magnitude of changes in gene expression tended to be smaller (data not shown). Analysis of the results was then begun (Task 4c).

c. *Data analysis of cDNA microarray data to identify putative downstream targets of* Gax. *(months 19-24).*

Status: In progress. We examined genes that were downregulated 24 hours after transduction of HUVECs with Ad.r*Gax* and were immediately struck by the number of CXC chemokines strongly downregulated (Table 1, which shows selected genes that are most strongly downregulated after *Gax* expression and/or most likely to be involved in angiogenesis). Most strongly downregulated of all was GRO-α (CXCL1), a CXC chemokine and a growth factor for melanoma that has also been implicated in promoting angiogenesis (72). Similarly, several other CXC chemokines were also strongly downregulated by *Gax* expression. Many of these peptides are clearly important in mediating EC activation during inflammation and in promoting angiogenesis (73). Consistent with the hypothesis that *Gax* inhibits EC activation, we also observed the downregulation of several cell adhesion molecules known to be upregulated in ECs during activation and angiogenesis, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin (74, 75). These proteins have all been implicated in leukocyte-EC interactions and are upregulated by pro-inflammatory factors and by VEGF during angiogenesis (74). The pattern of downregulation of these adhesion molecules, coupled with the downregulation of CXC chemokines, suggested to us inhibition of genes normally induced by TNF-α, which in turn suggested the possibility that *Gax* may inhibit nuclear factor κB (NF-κB) activity. Indeed, when we examined our data using GeneMAPP to look for patterns of signal-dependent gene regulation (76), we found numerous NF-κB-dependent genes (63) downregulated 24 hrs after *Gax* expression (Table 2).

The genes upregulated by *Gax* did not fall into any signal-dependent patterns as striking as the pattern of genes downregulated by *Gax* (Table 3). However, we did note results that might suggest specific pathways upregulated by *Gax*. First, there was a strong upregulation of ALK3 (bone morphogenetic receptor 1a) (77). Although it is known that, in ECs, ALK1 activates ECs through a SMAD1/5 pathway, whereas ALK5 inhibits EC activation through a SMAD2/3 pathway (66, 67), it is not known what role, if any, ALK3 plays in regulating EC phenotype. However, its upregulation by Gax implies that Gax may activate TGF-β signaling or render ECs more sensitive to TGF-β. Second, we noted the upregulation of three CDK inhibitors, $p19^{INK4D}$, $p57^{Kip2}$, and $p21^{WAF1/CIP1}$ (33, 61, 62), suggesting redundant mechanisms by which *Gax* can induce G_1 cell cycle arrest. Finally, we note that *Frizzled-2* was upregulated. Little is known about the potential role of *Frizzled* receptors and Wnt signaling in regulating postnatal angiogenesis, although *Frizzled-2* is known to be expressed in ECs and there is evidence suggesting Wnt signaling inhibits EC proliferation (64, 65). This data leads us to two potential other signaling pathways besides NF-kB to pursue in Years Two and Three.

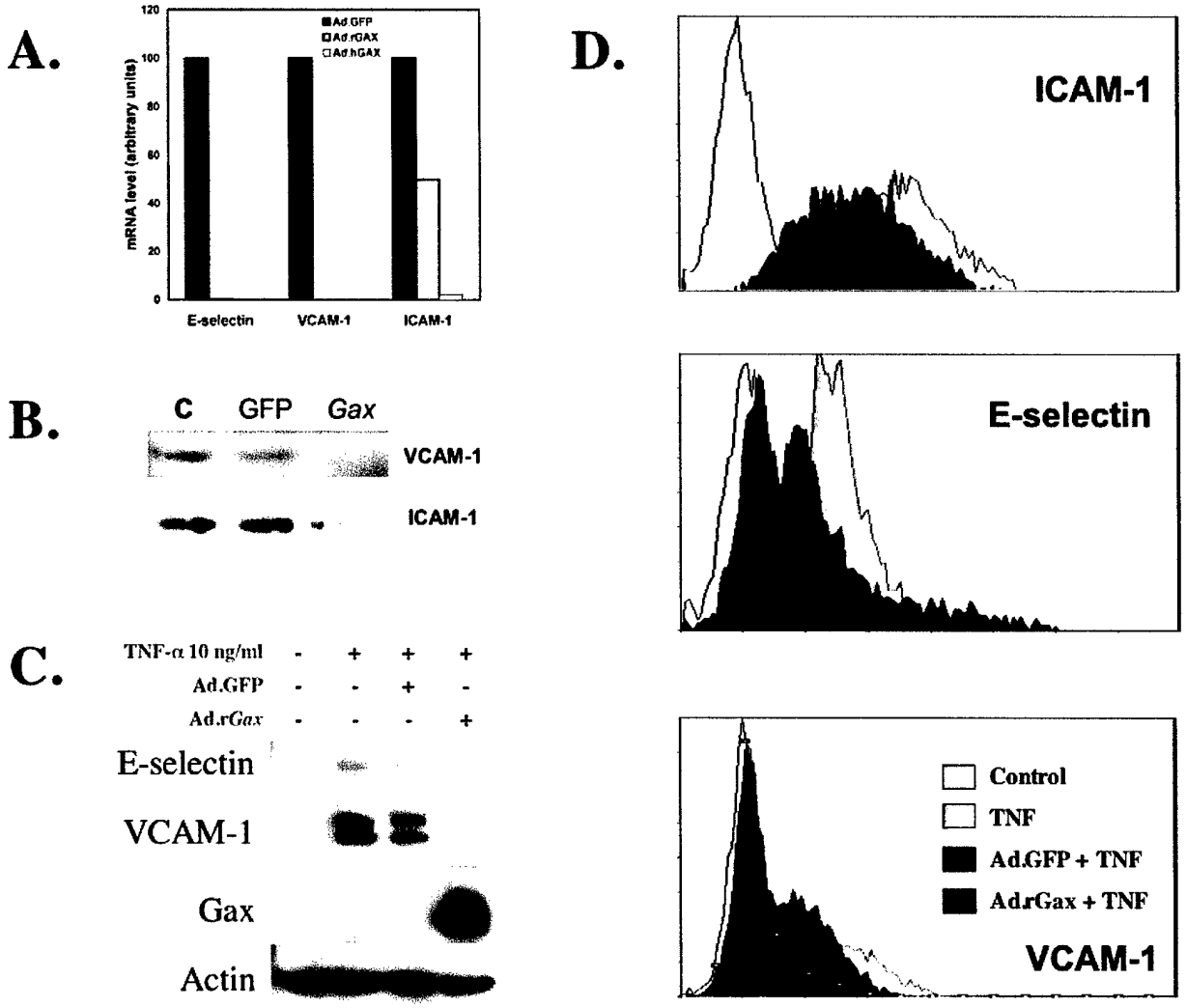

Figure 7. Effect of *Gax* expression on the level of E-selectin, VCAM-1, and ICAM-1. A. Quantitative real time PCR. Cells were harvested for total RNA isolation. Total RNA was then subjected to quantitative real time RT-PCR using TaqMan primers and probes specific for each gene and the results normalized to GAPDH. Units were chosen such that controls were set to 100. A very strong downregulation of E-selectin, VCAM-1, and ICAM-1 message level was observed. **B. *Gax* downregulates VCAM-1 and ICAM-1 proteins.** HUVECs were transduced with Ad.*rGax* or Ad.GFP and then incubated overnight. Cells were harvested for total protein and 50 μg protein was subjected to Western blot with appropriate antibodies. (C= control with no virus; GFP=Ad.GFP; *Gax*=Ad.*rGax*). E-selectin could not be visualized in unstimulated HUVECs. **C. *Gax* blocks upregulation of VCAM-1 and E-selectin.** HUVECs were transduced with Ad.*rGax* or Ad.GFP and then incubated overnight, after which they were stimulated with 10 ng/ml TNF-α for one hour. Cells were harvested for total protein and 50 μg protein was subjected to Western blot with appropriate antibodies. Expression of Gax from the adenoviral vector was verified by Western blot with antibodies against Gax previously described. **D. *Gax* downregulates cell surface expression of ICAM-1, E-selectin, and ICAM-1.** HUVECs transduced overnight with either Ad.GFP or Ad.*rGax* at an MOI=100 were stimulated with TNF-α 10 ng/ml for 4 hours and then harvested for flow cytometry using appropriate antibodies. Ad.r*Gax* blocked the expression of VCAM-1, E-selectin, and ICAM-1.

Task 5: Verification that putative downstream targets of Gax identified by cDNA microarray are regulated by Gax (months 19 through 36).

a. Real time quantitative PCR and Western blots of genes identified in Task 4 in order to verify regulation by Gax *(months 19-36).*

Status: In progress. Although we were not scheduled to start this part of the project until the second half of Year Two, given the results of the cDNA microarray experiments, we began to pursue the task of determining whether the genes identified on the array were truly downregulated by *Gax* expression. We have now verified that a number of the genes identified in the cDNA microarray experiments as being downregulated by *Gax* are also downregulated. First, we examined several NF-κB-dependent genes, because that would represent independent verification that NF-kB signaling pathways are downregulated by Gax expression. We found that basal and TNF-α-induced expression of ICAM-1, VCAM-1, and E-selectin were all strongly inhibited by Gax expression (Figure 7). This is consistent with a role for Gax in inhibiting NF-kB-dependent gene expression. In addition, we noted that proangiogenic peptides such as VEGF and bFGF were also downregulated, at least at the message level (Figure 8). These observations are suggestive of a role for Gax in not only blocking NF-kB-dependent gene activity but for potentially blocking angiogenesis through inhibition of the autocrine stimulation of ECs.

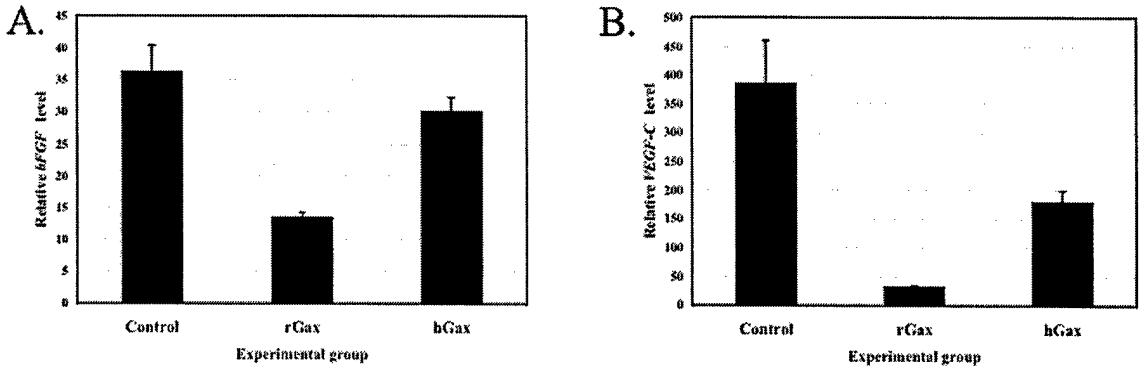

Figure 8. *Gax* downregulates proangiogenic factors expressed by ECs. HUVECs were transduced with either Ad.GFP (control), Ad.r*Gax*, or Ad.h*Gax* at MOI=100. After 24 hrs., cells were harvested for total RNA, which was then subjected to real time quantitative RT-PCR as described (Specific Aim 1). VEGF-C and bFGF message levels were normalized to GAPDH message. Units are arbitrary. **A.** bFGF. **B.** VEGF-C.

b. Analysis of the mechanism of regulation for the most strongly regulated genes (months 19-36).

Status: In progress. Given that NF-κB activity has been implicated in the changes in phenotype and gene expression ECs undergo during angiogenesis caused by VEGF, TNF-α, and other factors, and that a number of NF-κB targets have been implicated in inducing angiogenesis (78-84), we wished to confirm the finding from cDNA microarray studies that *Gax* inhibits NF-κB activity in ECs. We therefore performed EMSAs utilizing nuclear extracts from HUVECs transduced with either Ad.r*Gax* or the control adenoviral vector Ad.GFP to measure binding to a probe containing an NF-κB consensus sequence (85). Specific binding to NF-κB consensus sequence by nuclear extracts from HUVECs transduced with Ad.*Gax* and then induced with TNF-α (10 ng/ml) was much reduced compared to that observed in controls (Figure 9), implying

that *Gax* expression interferes with the binding of NF-κB to its consensus sequence. This suggests a method by which *Gax* may inhibit angiogenesis in breast cancer and an important hypothesis to test in Years Two and Three.

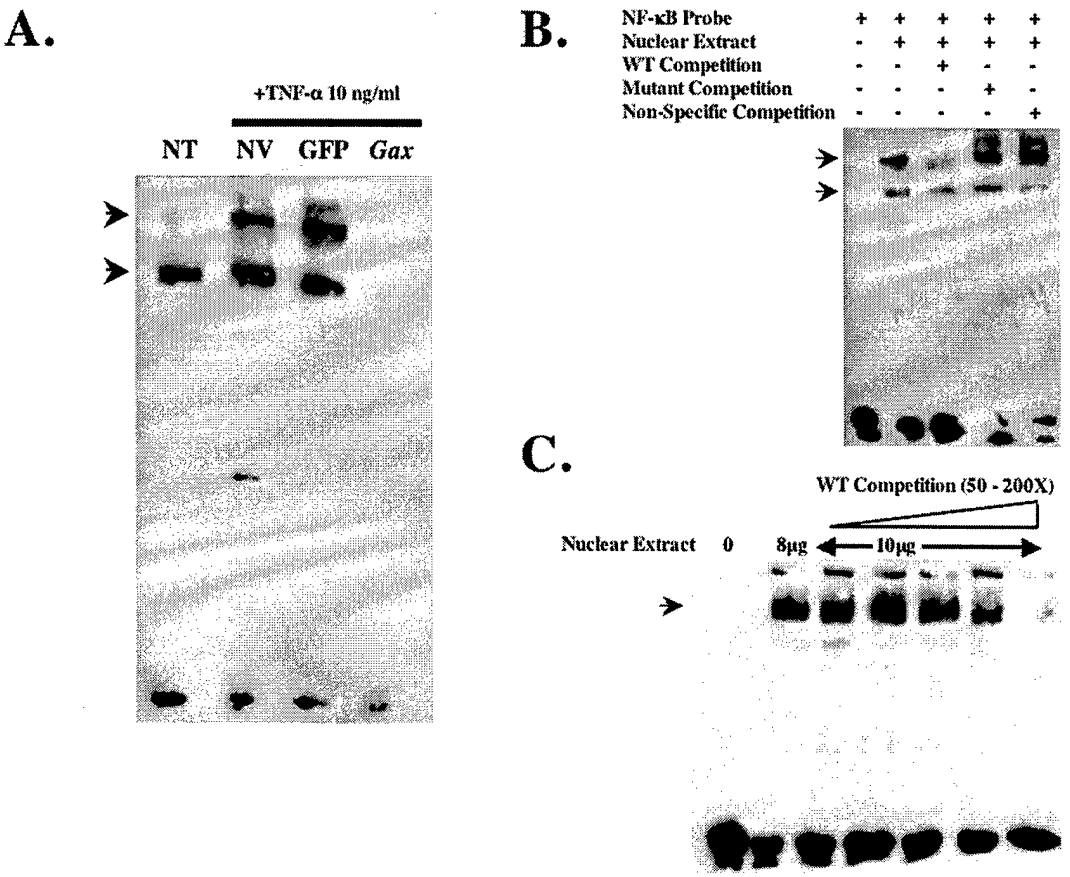

Figure 9. *Gax* expression inhibits NF-kB binding to its consensus sequence. A. Gax blocks NF-kB binding to its consensus sequence. HUVECs were infected with adenovirus containing GFP or r*Gax*, incubated overnight in EGM-2, and then induced with 10 ng/ml TNF-α for 1 hour. Controls were not induced with TNF-a. Nuclear extracts were prepared with the NE-PER nuclear extraction reagent (Pierce). Nuclear extracts were incubated with biotinylated oligonucleotides, containing the consensus NF-kB binding site, and the reactions were electrophoresed on a 6% acrylamide gel. The reactions were transferred to positively charged nylon membrane and detected with the LightShift EMSA kit (Pierce). Arrows denote NF-kB specific bands, and bands at the bottom of the gels represent unbound probe. **B and C. Control EMSAs.** These demonstrate failure of a random sequence oligonucleotide and an NF-kB consensus sequence with a point mutation that abolishes DNA binding to compete with wild-type NF-kB sequence **(B)** and competition with an excess of unlabeled wild-type NF-kB oligonucleotide **(C). Legend:** NT=no treatment; NV=no virus

KEY RESEARCH ACCOMPLISHMENTS

Our key research accomplishments during the past year include:

1. Demonstrated that mitogens and proangiogenic factors regulate *Gax* expression in ECs in a manner similar to that observed in vascular smooth muscle cells, with its expression maximal

in quiescent cells and rapidly downregulated after ECs are treated with mitogens, VEGF, or bFGF.

2. Demonstrated that proangiogenic factors secreted by breast cancer cells downregulate *Gax* expression in ECs.
3. Performed cDNA microarray experiments and began analysis of the data. This analysis shows that *Gax* downregulates the expression of NF-κB-dependent genes.
4. Confirmed cDNA microarray results for several genes identified in our initial cDNA microarray experiment at the message and protein level.
5. Demonstrated that *Gax* expression inhibits EC migration towards serum and proangiogenic stimuli.
6. Determined that *Gax* expression inhibits angiogenesis *in vivo* in the Matrigel plug assay.

REPORTABLE OUTCOMES

Abstracts

1. Patel, S., and **D. H. Gorski** (2004). Inhibition of endothelial cell activation and angiogenesis by the homeobox gene *Gax* is associated with downregulation of nuclear factor-κB (NF-κB)-dependent gene expression. *Proc. Amer. Assoc. Cancer Res.*45:77. Presented at the Annual Meeting of the American Association for Cancer Research, Orlando, FL, March 28, 2004

Journal articles:

1. **Gorski DH** and AD Leal (2003). Inhibition of endothelial cell activation by the homeobox gene *Gax*. *J. Surg. Res.* **111**: 91-99.
2. **Gorski DH**, and K Walsh (2003). Control of vascular cell differentiation by homeobox transcription factors. *Trends Cardiovasc Med* **13**: 213-220.
3. Patel S, Leal AD, and **DH Gorski** (2004). The homeobox gene *Gax* inhibits endothelial cell activation, angiogenesis, and nuclear factor kB activity. *Submitted, in review.*

Funding applied for based on work funded by DAMD17-02-1-0511:

Title/mechanism/role	Dates and funding	%Effort
1 R01 CA111344-01 **National Cancer Institute** **Role: Principal investigator**	1/1/2005 – 12/31/2009	40% effort

Mechanism of angiogenesis inhibition by a homeobox gene

Status: Under review.

CONCLUSIONS

Homeobox genes are master regulatory genes with diverse functions in many cell types, both during embryogenesis and in the adult (1, 3, 4, 6, 86). It is therefore not surprising that recently they have been implicated as important transcriptional regulators controlling endothelial cell phenotype during tumor-induced angiogenesis (7, 8, 12, 13, 52, 55). Until recently, little was known about how homeobox genes might influence endothelial cell phenotype and behavior

during breast cancer-induced angiogenesis. However, evidence for their involvement in the phenotypic changes endothelial cells undergo during angiogenesis is now accumulating. For instance, Patel *et al* reported an endothelial cell-specific variant of *HOXA9* whose expression is regulated by tumor necrosis factor-α, which is proangiogenic (87). More direct evidence for the importance of homeobox genes in angiogenesis exists for *HOXD3* (7). *In vivo*, sustained expression of *HOXD3* on the chick chorioallantoic membrane (CAM) retains endothelial cells in an invasive state and prevents vessel maturation, leading to vascular malformations and endotheliomas. In diabetic mice, *HOXD3* expression is impaired in endothelial cells, as is its upregulation after wounding (52). Moreover, HOXD3 expression is elevated in breast cancer tumor vasculature as compared to normal vasculature, as measured by *in situ* hybridization (9). More recently, overexpression of another homeobox gene, *HOXB3* has been shown to result in an increase in capillary vascular density and angiogenesis, and its blockade by antisense results in impaired capillary morphogenesis (8). In contrast, *HOXD10* inhibits EC conversion to the angiogenic phenotype, and sustained expression of *HOXD10* inhibits EC migration and blocks bFGF- and VEGF-induced angiogenesis *in vivo* (88). Consistent with this, *HOXD10* expression is decreased in breast cancer vasculature (12). Another homeobox gene, *Hex,* has a more complex role, being upregulated during angiogenesis but inhibiting EC tube formation on basement membranes (13). When combined with previous data showing high levels of *Hex* expression in proliferating vasculature had suggested that *Hex* would be more likely to induce EC proliferation and angiogenesis (54, 55), the observation that *Hex* inhibits *in vitro* angiogenesis suggests a more complex role for this gene than previously understood. Taken together, these data suggest significant roles for specific homeobox genes in responding to extracellular signals and activating batteries of downstream genes to induce or inhibit the phenotypic changes in endothelial cells associated with angiogenesis. These observations are what initially led us to look for additional homeobox genes likely to be involved in the final transcriptional control of genes determining angiogenic phenotype in breast cancer. Because blocking aberrant angiogenesis has the potential to be an effective strategy to treat or prevent multiple diseases,, understanding how downstream transcription factors integrate upstream signals from pro- and anti-angiogenic factors to alter global gene expression and produce the activated, angiogenic phenotype, will be increasingly important in developing effective antiangiogenic therapies for breast cancer.

Based on our data, we postulated that at least one additional homeobox gene, *Gax*, is also likely to have an important role in the phenotypic changes that occur in ECs during angiogenesis and therefore wanted to study its role in regulating breast cancer-induced angiogenesis. We originally isolated *Gax* from a rat aorta library (15), and subsequently we and others found that in the adult its expression is restricted primarily to mesodermal tissues, particularly the cardiovascular system (14, 16, 18). Moreover, *Gax* expression is rapidly downregulated by growth factors and more slowly upregulated by growth arrest signals in VSMCs both *in vitro* and *in vivo* (15, 30, 32), and its expression results in cell cycle arrest (14, 33), p21 induction (14, 33), inhibition of migration (31), and modulation of integrin expression (31). *In vivo*, *Gax* expression in injured vasculature prevents the proliferative response that leads to restenosis after balloon angioplasty (22, 23, 26, 33). Based on these observations, we examined Gax expression in vascular ECs. We found that *Gax* is expressed in this cell type and that it has many of the same activities as in VSMCs. In addition, its expression inhibited EC tube formation on Matrigel *in vivo* (14). These observations led us to the present study, in which we wished to elucidate further the role(s) *Gax* may have in regulating angiogenesis, in particular breast cancer-induced

angiogenesis. Consistent with its regulation in VSMCs, in ECs, *Gax* is rapidly downregulated by serum, proangiogenic, and pro-inflammatory factors (Figures 1 and 2), and is able to inhibit EC migration in vitro (data not shown) and angiogenesis *in vivo* (Figure 5) These observations led us to examine the mechanism by which *Gax* inhibits EC activation utilizing cDNA microarrays to examine global changes in gene expression due to *Gax*. In addition to observing that Gax upregulates cyclin kinase inhibitors (Table 3) and downregulates a number of proangiogenic factors (Tables 1 and 2), we also found that Gax inhibits the expression of a number of NF-κB target genes (Table 2). Consistent with the cDNA microarray data, *Gax* inhibits the binding of NF-κB to its consensus sequence (Figure 8).

The NF-κB/Rel proteins are an important class of transcriptional regulators that play a central role in modulating the immune response and promoting inflammation and cancer by regulating the expression of genes involved in cell growth, differentiation, and apoptosis. In many cell types, NF-κB promotes cell survival in response to pro-apoptotic stimuli, induces cellular proliferation, or alters cell differentiation. The NF-κB/Rel family is composed of at least five mammalian homologs, c-Rel, RelA (p65), RelB, p50/p105 (NF-κB1), and p52/p100 (NF-κB2), which form an array of homo- and heterodimers, known as the NF-κB complex (63). In most cell types, NF-κB exists in the cytoplasm as an inactive form bound to inhibitory proteins known as IκBs. In the classical pathway of NF-κB activation, NF-κB inducers, such as TNF-α and other proinflammatory cytokines, growth factors, UV light, oxidative stress, and bacterial lipopolysaccharide, initiate a signaling cascade ultimately leading to the nuclear translocation of p50/RelA heterodimers, resulting from signal-induced phosphorylation of IκB by IκB kinase (IKK), which targets it for ubiquitination (63, 89). In addition to this classical pathway of NF-κB activation, a non-canonical pathway involving activation of p52-containing dimmers through regulated processing of the p100 precursor protein (63, 89).

Several lines of evidence have implicated NF-κB activity in regulating EC phenotype during inflammation and angiogenesis and, in particular, the classic activation of RelA-containing heterodimers (74, 78-83, 90). For example, proangiogenic factors such as VEGF (74), TNF-α (90), and platelet-activating factor (78) can all activate NF-κB signaling and activity in ECs. In addition, inhibition of NF-κB activity inhibits EC tube formation *in vitro* on Matrigel (83, 91), and pharmacologic inhibition of NF-κB activity suppresses retinal neovascularization *in vivo* in mice. (92) Moreover, ligation of EC integrin $\alpha_V\beta_3$ by osteopontin protects ECs against apoptosis induced by serum withdrawal, an effect that is due to NF-κB-dependent expression of osteoprotogerin (81). Similarly, $\alpha_5\beta_1$-mediated adhesion to fibronectin also activates NF-κB signaling and is important for angiogenesis, and inhibition of NF-κB signaling inhibits bFGF-induced angiogenesis (79). One potential mechanism by which NF-κB signaling may promote angiogenesis is through an autocrine effect, whereby activation of NF-κB induces expression of proangiogenic factors such as VEGF, as has been reported for platelet-activating factor-induced angiogenesis (78). Alternatively, the involvement of NF-κB in activating EC survival pathways is also likely to be important for sustaining angiogenesis (91).

Although NF-κB activity can influence the expression of homeobox genes (87, 93), there have been relatively few reports of functional interactions between homeodomain-containing proteins and NF-κB proteins. The first such interaction reported was between IκBα and HOXB7, where IκBα was found to bind through its ankyrin repeats to the HOXB7 protein and potentiate

HOXB7-dependent gene expression (94). More recently, it was reported that IκBα can also potentiate the activity of other homeobox genes, including *Pit-1* and *Pax-8*, through the sequestration of specific histone deacetylases (95). In contrast, Oct-1 can compete with NF-κB for binding to a specific binding site in the TNF-α promoter (96). In addition, at least one interaction has been described in which a homeobox gene directly inhibits NF-κB-dependent gene expression, an interaction in which Cdx2 blocks activation of the COX-2 promoter by binding p65/RelA (97). It remains to be elucidated if *Gax* inhibits NF-κB-dependent gene expression by a similar mechanism. Regardless of the mechanism, however, our observations made while doing the research funded by this Idea Award, to our knowledge, represent the first description of a homeobox gene that not only inhibits phenotypic changes that occur in ECs in response to proangiogenic factors, but also inhibits NF-kB-dependent gene expression in vascular ECs. These properties suggest *Gax* as a potential target for the antiangiogenic therapy of breast cancer. In addition, understanding the actions of *Gax* on downstream target genes, signals that activate or repress *Gax* expression, and how *Gax* regulates NF-κB activity in ECs is likely to lead to a better understanding of the mechanisms of breast cancer-induced angiogenesis and the identification of new molecular targets for the antiangiogenic therapy of breast cancer.

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APPENDICES

Publications during period of report:

1. **Gorski DH** and AD Leal (2003). Inhibition of endothelial cell activation by the homeobox gene *Gax*. *J. Surg. Res.* **111**: 91-99.
2. **Gorski DH** and K Walsh (2003). Control of vascular cell differentiation by homeobox transcription factors. *Trends Cardiovasc Med* **13**: 213-220.
3. Patel, S, Leal, AD, and **DH Gorski** (2004). The homeobox gene *Gax* inhibits endothelial cell activation, angiogenesis, and nuclear factor kB activity. *Submitted.*

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Inhibition of Endothelial Cell Activation by the Homeobox Gene *Gax*

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Background. **Angiogenesis is critical to tumor growth. *Gax*, a homeobox transcription factor whose expression in the adult is restricted mainly to the cardiovascular system, strongly inhibits growth factor-stimulated phenotypic modulation of vascular smooth muscle cells *in vitro* and *in vivo*. The function of *Gax* in vascular endothelium is unknown, but we hypothesized that it may play a similar role there. We therefore studied *Gax* expression in vascular endothelial cells and its effects on proliferation and tube formation.**

Materials and methods. ***Gax*** **expression in normal endothelial cells was examined *in vitro* by Northern blot and reverse transcriptase polymerase chain reaction and *in vivo* by immunohistochemistry. A replication-deficient adenovirus was then used to express *Gax* in human umbilical vein endothelial cells (HUVECs). HUVEC proliferation, ^{3}H-thymidine uptake, p21 expression, and tube formation on reconstituted basement membrane were measured at different viral multiplicities of infection.**

Results. ***Gax*** **mRNA was detected in HUVECs by reverse transcriptase polymerase chain reaction and Northern blot analysis and in normal vascular endothelium by immunohistochemistry. Compared with controls transduced with a virus expressing β-galactosidase, *Gax* strongly inhibited HUVEC proliferation and mitogen-stimulated ^{3}H-thymidine uptake. p21 expression in HUVECs transduced with *Gax* was increased up to 5-fold as measured by Northern blot, and p21 promoter activity was activated by 4- to 5-fold. Tube formation on Matrigel was strongly inhibited by *Gax* expression.**

Conclusions. ***Gax*** **is expressed in vascular endothelium and strongly inhibits endothelial cell activation in response to growth factors and tube formation *in vitro*. These observations suggest that *Gax* inhibits endothelial cell transition to the angiogenic phenotype in response to proangiogenic growth factors and, as a negative regulator of angiogenesis, may represent a target for the antiangiogenic therapy of cancer.**

Key Words: **angiogenesis; homeobox genes; transcription factors; vascular endothelium.**

INTRODUCTION

Vascular remodeling plays a critical role in the biology of tumors, whose growth without a blood supply is limited to less than 1 mm in diameter by diffusion of oxygen and nutrients through the interstitial fluids [1]. To overcome this limitation, tumors secrete proangiogenic factors, such as vascular endothelial growth factor (VEGF) [2] and basic fibroblast growth factor (bFGF) [3], to stimulate the ingrowth of new blood vessels [1, 4]. To form new tumor vasculature, endothelial cells undergo profound phenotypic changes, many of which are similar to the phenotypic changes tumor cells undergo when invading the surrounding stroma [1, 5, 6]. They degrade their basement membrane and invade the surrounding tissue, migrate towards the proangiogenic stimulus secreted by the tumor, and then form tubular structures and finally neovasculature [1, 7]. Although the receptors and signaling pathways activated by proangiogenic factors and cytokines have been extensively studied in endothelial cells [8, 9], much less is known about the molecular biology of the downstream transcription factors that regulate the tissue-specific gene expression controlling endothelial cell growth and differentiation and are activated by these signaling pathways. These transcription factors represent a common mechanism that can be influenced by the interaction of multiple signal-

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ing pathways and therefore might represent targets for the antiangiogenic therapy of cancer.

To understand the transcriptional control of tumor-induced angiogenesis and thereby potentially identify new ways to target it therapeutically, we decided to study the role of homeobox transcription factors in regulating the phenotypic changes that occur in endothelial cells when stimulated with proangiogenic factors. Because of their ubiquitous role as regulators of cell proliferation, migration, and differentiation, as well as body plan formation and organogenesis during embryogenesis in vertebrates and invertebrates [10, 11] and as oncogenes and tumor suppressors in various human cancers [12, 13], of all the various classes of transcription factors, we considered homeobox genes as especially likely to be important in regulating endothelial cell phenotype during angiogenesis.

Among homeobox genes, *Gax* (*G*rowth *A*rrest-specific homeobo*X*) has several characteristics that suggest it as a candidate for a role as an inhibitor of the endothelial cell phenotypic changes that occur as a result of stimulation by proangiogenic factors. Originally isolated from vascular smooth muscle [14], in the adult *Gax* expression is largely restricted to the cardiovascular system [14, 15]. In vascular smooth muscle cells, *Gax* expression is downregulated by mitogens [14, 16] and upregulated by growth arrest signals [14, 17]. Consistent with this observation, *Gax* expression induces G_1 cell cycle arrest [18] and inhibits vascular smooth muscle cell migration, downregulating the expression of integrins, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [19], both of which are associated with the synthetic state in vascular smooth muscle cells and the angiogenic phenotype in endothelial cells [19, 20]. *In vivo, Gax* expression in arteries inhibits proliferative restenosis of the arterial lumen after injury [21]. Because *Gax* expression is largely confined to the cardiovascular system and mesoderm-derived structures [15, 22], we considered it likely that *Gax* is also expressed in endothelial cells because endothelial cells are also derived from mesoderm. Because of its activities in vascular smooth muscle cells, we further hypothesized that *Gax* may be involved in inhibiting the phenotypic changes that occur in endothelial cells in response to stimulation with proangiogenic factors. In this report, we show that *Gax* is also expressed in vascular endothelial cells and inhibits endothelial cell cycle activation and tube formation in response to proangiogenic factors, suggesting that it has a role as a negative regulator of angiogenesis.

MATERIALS AND METHODS

Cells and Cell Culture

Human umbilical vein endothelial cells were obtained from Cambrex Biosciences (Walkersville, MD) and cultured as previously described [23] according to manufacturer's instructions in EGM-2 medium (Cambrex Biosciences, Walkersville, MD). For experiments, recombinant $VEGF_{165}$ (R & D Systems, Minneapolis, MN) was substituted in the media at the concentrations indicated for the proprietary VEGF solution.

Plasmid and Adenoviral Constructs

The *Gax* cDNA was maintained in pBluescript SK+ vectors and excised as needed for use as probes for Northern blots. Adenoviral constructs expressing the human and rat homologs of *Gax* (Ad.*hGax* and Ad.*rGax*, respectively) conjugated to the α-hemagluttinin (HA) epitope were a kind gift of Dr. Kenneth Walsh (Boston University, Boston, MA) [18], as was the control adenoviral vector expressing β-galactosidase (Ad.*β-Gal*). Both human and rat isoforms of Gax were used to verify that both isoforms have similar activity. The control adenoviral vector expressing green fluorescent protein (Ad.*GFP*) was a kind gift of Dr. Daniel Medina (The Cancer Institute of New Jersey, New Brunswick, NJ). Viral titers were determined by plaque assay. Prior to the use of Ad.*hGax* or Ad.*rGax* in HUVECs, expression of *Gax* mRNA and protein in cells transduced with these adenoviral constructs were verified by Northern and Western blot (not shown). The p21 cDNA and p21 promoter constructs were also obtained from Dr. Kenneth Walsh and are the same constructs used in other studies [18]. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA used as a probe for Northern blots was the same construct used in another study [14].

Immunohistochemistry

Tissue sections were obtained from human surgical specimens and fixed and imbedded in paraffin according to standard procedures, with sections dehydrated through xylenes and then rehydrated through graded ethanols [15]. Staining with a polyclonal rabbit anti-Gax antibody, which labels rat, human, and mouse Gax protein, was performed according to previously described methods, except that the dilution used was 1:1000 [15]. A biotin-labeled goat anti-rabbit IgG (Sigma Corporation, St. Louis, MO) was used as a secondary antibody, and Gax staining was visualized using Vectastain ABC (Vector Laboratories, Burlingame, CA). Background staining was assessed by staining sections without primary antibody. All tissue specimens were obtained from a protocol approved by the Institutional Review Board of the University that protects the privacy of the patients from which the samples were obtained.

Northern Blots

Northern blots measuring *Gax* expression were performed as previously described [14]. Briefly, total RNA (30 μg) was isolated from cultured cells using the guanidinium thiocyanate method [24] subjected to electrophoresis through formaldehyde-containing agarose gels, capillary blotted to nylon membranes using 10× SSC as the transfer buffer, fixed to the membrane using ultraviolet crosslinking, and then hybridized to the *Gax* cDNA labeled with ^{32}P by random priming in Church buffer [25]. Blots were exposed to Kodak XAR-5 X-ray film with an intensifying screen at −80° C. Blots were then stripped with 0.1× SSC plus 0.1% SDS at 95°C and reprobed with the GAPDH cDNA to verify equal RNA loading. Hybridization temperatures were 55°C for *Gax*, p21, and GAPDH probes, and all blots were washed to a stringency of 0.2× SSC at 65°C. For p21 Northern blots, autoradiographs were scanned and band intensities determined with NIH Image v.1.6 p21 message levels were then normalized to GAPDH levels, and the fold-induction of p21 determined.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA was isolated as described above from HUVECs and used in RT-PCR to detect *Gax* transcripts. Total RNA (5 μg) was subjected to

reverse transcriptase reaction with MMLV-reverse transcriptase (Invitrogen, Carlsbad, CA) using random hexamers (Invitrogen, Carlsbad, CA). Because *Gax* has a single exon [26], all samples were treated with RNAse-free DNAse I (Ambion, Austin, TX) before being subjected to reverse transcription. As a further means of verifying that there was no genomic DNA contamination, control reactions with no reverse transcriptase were also subjected to PCR. To check the integrity of the RNA, the same reverse transcriptase reactions used to detect *Gax* were subjected to PCR using β-actin–specific primers. Human *Gax* primer sequences were: 5′-GTCAGAAGTCAACAGCAAACCCAG-3′, sense; 5′-CACATTCACCAGTTCCTTTTCCCGAGCC-3′, antisense; product size 247 bp, from nucleotides 566 to 812 (26). Human β-actin primer sequences were: 5′-ATCCGCAAAGACCTGT-3′, β-actin sense; and 5′-GTCCGCCTAGAAGCAT-3′ β-actin antisense; product size 270 bp, from nucleotides 906 to 1175 [27]. Before *Gax* primers were synthesized, their sequences were subjected to a BLAST [28] search against the Genbank database to detect any possibility that they might bind to or amplify genes other than *Gax*. Before running assays on experimental samples, each primer set, annealing conditions, Mg^{2+} concentration, and primer and probe concentration were optimized using plasmids containing the cDNA of interest. Reaction mixtures (25 μl) were used containing 0.75 U *Taq* polymerase (Gibco BRL), reaction buffer, 0.2 mM dNTPs, plus the optimized concentrations of $MgCl_2$, probe, and primers for each primer set. The PCR cycle consisted of an initial 5-min denaturation step at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C (*Gax*) or 54°C (β-actin) for each primer for 60 s, and extension at 72°C for 60 s.

Cell Proliferation and ^{3}H-Thymidine Incorporation

The effect of *Gax* overexpression on mitogen-stimulated ^{3}H-thymidine incorporation was examined in HUVECs. For cell proliferation, randomly cycling HUVECs in 6-well plates (20,000 cells/plate) were transduced for 12 h with Ad.*Gax* or Ad.*β-gal* at varying MOIs, after which they were washed 3 times with phosphate-buffered saline and then placed in fresh medium EGM-2 supplemented with 10 ng/ml $VEGF_{165}$). After infection, every day 3 wells for each experimental group were trypsinized and viable cells counted, with cell viability determined by Trypan blue exclusion. For ^{3}H-thymidine uptake studies, HUVECs were made quiescent by serum starvation for 24 h in medium containing 0.1% fetal bovine serum (FBS) at which point the cells were transduced with Ad.*Gax* or Ad.*βGal* and incubated in 0.1% FBS for an additional 24 h. The cells were then stimulated with medium containing 10% FBS and 10 ng/ml $VEGF_{165}$ for 24 h in the presence of 0.2 μCi/ml ^{3}H-thymidine (Amersham, Piscataway, NJ), after which trichloroacetic acid precipitable counts were measured.

Transactivation of the p21 Promoter

Subconfluent HUVECs were plated in 6-well plates and allowed to attach for 4 h. They were then infected with different MOIs of Ad.*hGax*, Ad.*r*Gax, or Ad.*GFP* overnight, then transfected with p21 promoter Luciferase reporter construct. Transfection was performed using 2 μg p21-Luciferase plasmid per well, plus 0.2 μg pRL-SV (Promega, Madison, WI), which contains the cDNA for *Renilla reniformis* Luciferase downstream from the SV40 promoter as its reporter instead of the cDNA for firefly Luciferase, as a control for transfection efficiency. Firefly and *Renilla* Luciferase activities were measured using the Dual Luciferase Assay Kit (Promega, Madison, WI), and the firefly Luciferase activity from the p21-Luciferase promoter construct normalized to the constitutive *Renilla* Luciferase activity from the pRL-SV plasmid.

Tube Formation Assay

Tube formation assays were performed essentially as described [29]. Briefly, HUVECs were infected with adenoviruses expressing either human *Gax* (Ad.*hGax*), rat *Gax* (Ad.*rGax*), or GFP (Ad.*GFP*) at various multiplicity of infection (MOI). Eighteen hours later 5×10^5 cells were plated on 6 well plates whose surfaces had been coated with reconstituted basement membrane, Low Growth Factor Matrigel, (BD Biosciences, San Jose, CA) and incubated overnight in the presence of serum and 10 ng/ml $VEGF_{165}$. After this, the number of tubes per high-powered field were counted for 10 high-powered fields, with tubes being defined as a completed connection between cells. Ad.GFP-transduced cells were also examined using a fluorescence microscope to demonstrate that GFP was being expressed in the HUVECs forming tubes.

Data Analysis and Statistics

Experiments were repeated 3 or more times. For cell culture experiments, at least three wells per experimental group were measured and the mean ± standard deviation determined. Statistical significance between the various groups was determined by 2-way ANOVA and the appropriate post-test, with the results being considered statistically significant when $P < 0.05$.

RESULTS

Gax is Expressed in Human Vascular Endothelium

Because we hypothesized that Gax is expressed in endothelial cells as well as vascular smooth muscle cells, we first examined *Gax* expression in cultured human vascular endothelial cells and detected *Gax* expression in HUVECs by Northern blot (Fig. 1A) and by RT-PCR using human *Gax*-specific primers (Fig. 1B). Next, to verify that Gax protein is expressed in the endothelium of normal human blood vessels, we subjected a section of human kidney from a nephrectomy specimen to immunohistochemistry with a polyclonal rabbit anti-Gax antibody [15] (Fig. 2). As expected, *Gax* was expressed in vascular smooth muscle cells. In addition, it was also expressed in the endothelial cells lining the lumen of arteries, as evidenced by nuclear staining of the cells of the intima. From these observations, we conclude that *Gax* is expressed in normal endothelial cells, both *in vitro* and *in vivo*.

Gax Inhibits HUVEC Proliferation *in Vitro*

To test the hypothesis that *Gax* expression inhibits proliferation of endothelial cells, we transduced HUVECs that had been sparsely plated on plastic in 6-well plates with Ad.*hGax* at increasing MOI. Viable cells were counted from each experimental group every 24 h for 4 days. Control cells were transduced with Ad.*β-gal*. Up to MOI = 1000, Ad.*β-gal* did not inhibit HUVEC proliferation (data not shown). Both Ad.*hGax* and Ad.*rGax*, however, inhibited HUVEC and proliferation in a dose-dependent fashion compared to Ad.*β-gal* (Fig. 3A and B; $P < 0.05$ for all MOI of virus). Quiescent HUVECs were then transduced with either

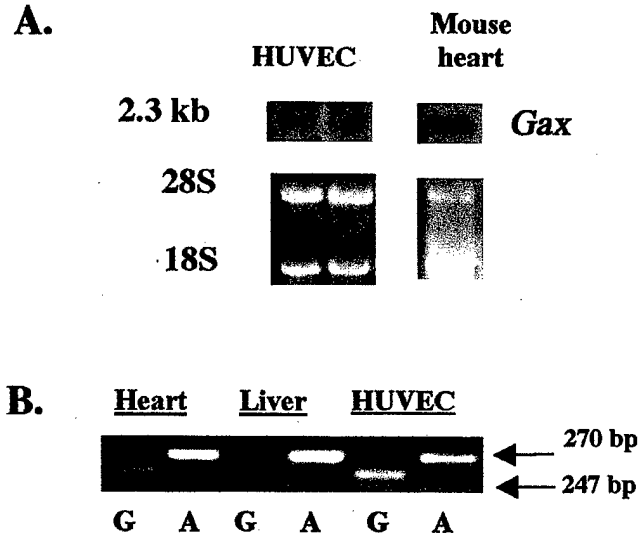

FIG. 1. Gax expression in vascular endothelial cells. Total RNA from HUVECs was subjected to Northern blot with the *Gax* cDNA labeled with ^{32}P by random priming. (A) Northern blots. Two different HUVEC preparations were studied and compared to mouse heart (MH), which is known to express *Gax*. (B) RT-PCR. Total RNA from HUVECs was subjected to RT-PCR using primers that amplify a 247-bp fragment (base 566 to 812) of the human *Gax* cDNA. The same RT reactions were also subjected to PCR using β-actin primers. See Materials and Methods for details. (G = *Gax*; A = β-actin).

Ad.*hGax* or Ad.*β-gal*, maintained in low serum medium for 24 h, then stimulated with 10% FBS and $VEGF_{165}$ = 10 ng/ml, and 24-h ^3H-thymidine uptakes measured (Fig. 4). For comparison, one experimental group was left in low serum medium and is labeled "Quiescent." Consistent with its effect on randomly cycling HUVECs. *Gax* strongly inhibited mitogen-stimulated ^3H-thymidine uptake ($P < 0.05$ for all MOI of virus). From these results, we conclude that *Gax* expression results in inhibition of HUVEC proliferation, as well as cell cycle arrest.

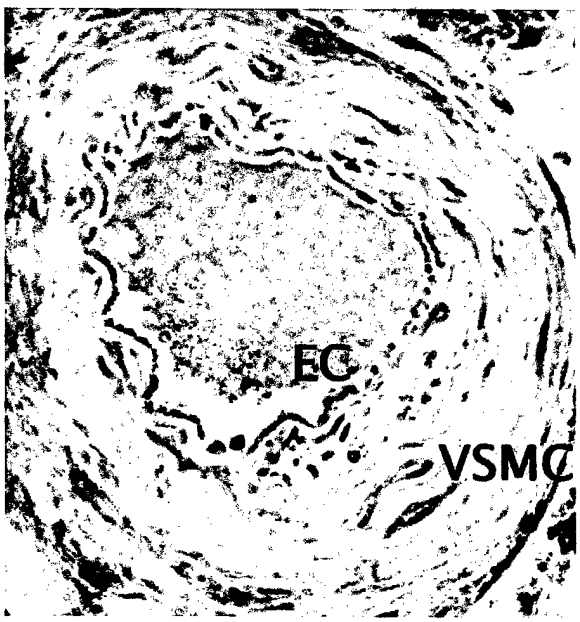

FIG. 2. *Gax* is expressed in both the vascular smooth muscle cells and the endothelial cells of normal human arteries. A section from human kidney obtained from a nephrectomy specimen for renal cell carcinoma was stained with rabbit polyclonal anti-Gax antibody. In the section containing normal kidney, Gax expression was noted in both the media, containing vascular smooth muscle cells (VSMC), as expected from previous studies, but there was also strong staining in the endothelial cells (EC) in the intima lining the lumen.

Gax Activates p21 Promoter Activity in Endothelial Cells

Because *Gax* induces p21 in vascular smooth muscle cells and *Gax* expression inhibited HUVEC proliferation as measured both by cell counts and ^3H-thymidine uptake, we tested whether *Gax* could induce p21 expression in endothelial cells. HUVECs were transduced with Ad.*hGax* and Ad.*rGax* at varying MOIs. Cells transduced with an adenovirus expressing green fluorescent protein (Ad.*GFP*) served as controls. By Northern blot, p21 levels were strongly induced in a viral MOI-dependent fashion (Fig. 5A). When cells transduced with Ad.*hGax* in a similar fashion were transfected with a plasmid containing the p21 promoter fused upstream to the firefly Luciferase gene, it was similarly observed that p21 promoter activity was increased by up to 7-fold (Fig. 5B; $P < 0.05$ for all MOI). Transduction with Ad.*GFP* did not affect p21 promoter activity (Fig. 5A and B), nor did transduction with Ad.*β-Gal* (data not shown).

Gax Inhibits Endothelial Cell Tube Formation on Reconstituted Basement Membranes

We next studied the effect of *Gax* expression on angiogenesis *in vitro*. HUVECs were transduced with

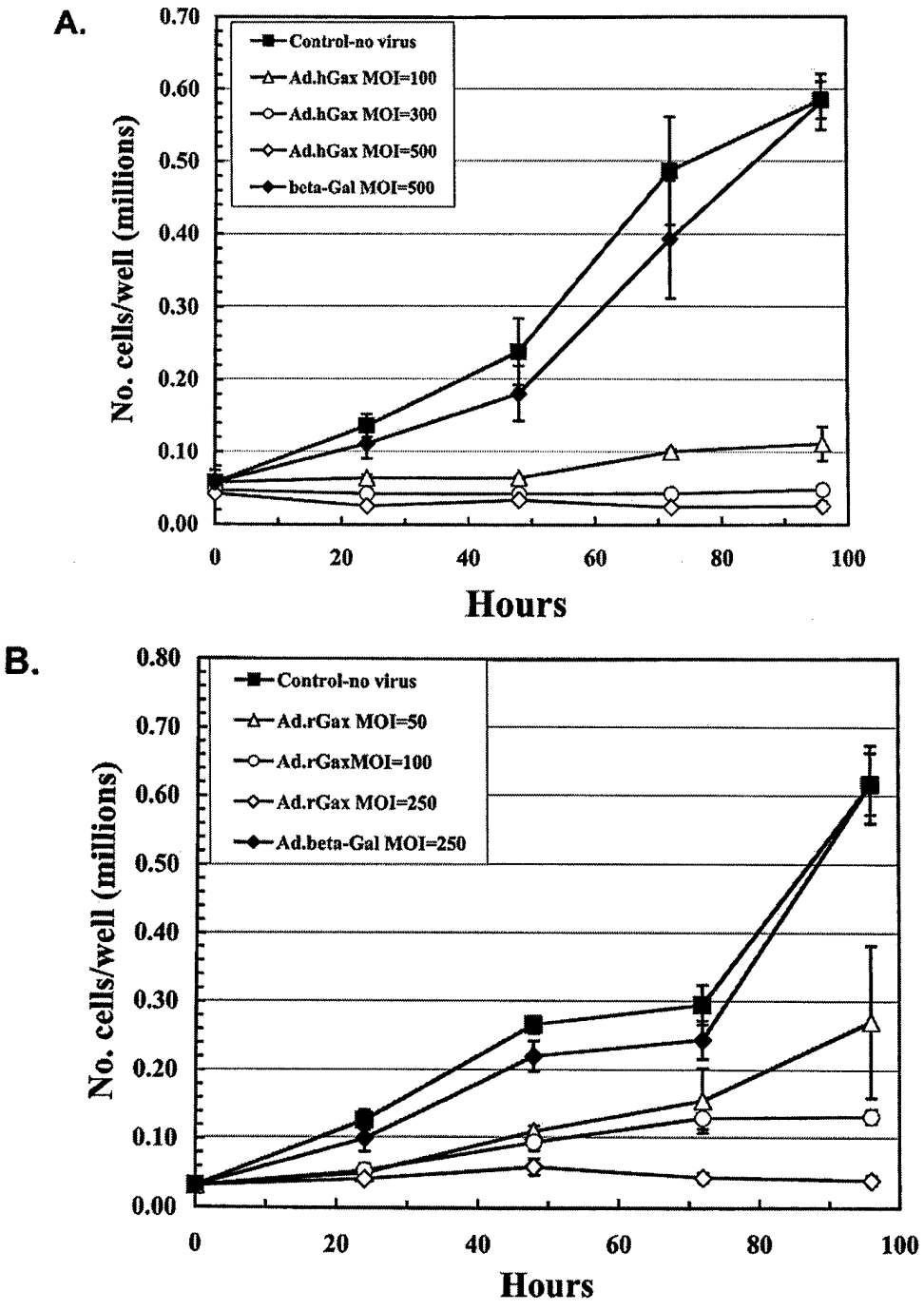

FIG. 3. Inhibition of HUVEC proliferation by *Gax*. Randomly cycling HUVECs growing in 6-well plates in EGM-2 medium were infected with varying MOI of either Ad.*hGax*, Ad.*rGax*, or Ad.*β-Gal*. After infection, 3 wells for each experimental group were trypsinized and counted, with cell viability determined by Trypan blue exclusion, and results were counted as mean number of cells ± standard deviation. Inhibition of proliferation was statistically significant for all experimental groups at all time points from 48 hours on ($P < 0.05$). (A) Effect of Ad.*hGax* on HUVEC proliferation (B) Effect of Ad.*rGax* on HUVEC proliferation.

Ad.*hGax* and Ad.*rGax* at varying MOIs and plated on reconstituted basement membrane (Matrigel) in the presence of serum and 10 ng/ml $VEGF_{165}$, conditions that result in robust tube formation. Ad.*GFP* had no effect on tube formation up to MOI = 250, and expression of GFP was verified by fluorescence microscopy (Fig. 6). However, there was a dose-dependent decrease in tube formation beginning at relatively small doses of virus (MOI = 25) and becoming maximal at MOI = 100 (Fig. 6). Maximal inhibition oc-

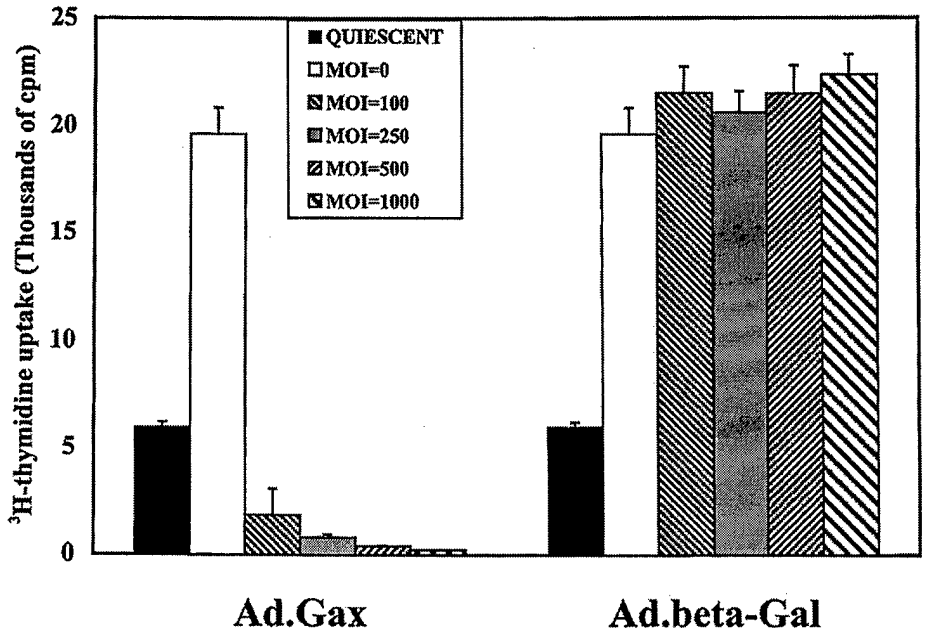

FIG. 4. Inhibition of mitogen-induced ^{3}H-thymidine uptake in HUVECs by *Gax*. Quiescent HUVECs were transduced with Ad.*hGax* at various MOI. Twenty-four hours later, the cells were stimulated with serum and $VEGF_{165}$ (10 ng/ml) and 24 h. ^{3}H-thymidine uptakes measured after stimulation. *Gax* strongly inhibited ^{3}H-thymidine uptake in response to mitogen stimulation.

curred at a lower MOI than is necessary to maximally inhibit endothelial cell proliferation and activate p21 expression and became maximal at MOI = 50 to 100. We note that is the dose range of virus that we have determined to be necessary to transduce 100% of HUVECs (not shown), implying that few viral particles per cell are necessary to produce sufficient Gax protein to inhibit the cellular machinery that causes tube formation. This is in contrast to the higher viral MOI necessary to produce maximal inhibition of cell cycle progression and induction of p21 expression, implying that more viral particles per cell and therefore a higher level of Gax protein are required to mediate these effects.

DISCUSSION

The primary target of proangiogenic factors secreted by tumor cells, and many antiangiogenic factors, is the vascular endothelial cell [1, 30]. During angiogenesis, whether physiologic or tumor-induced, endothelial cells undergo distinct changes in phenotype and gene expression, including activation of proteolytic enzymes to degrade basement membrane, sprouting, proliferation, tube formation, and production of extracellular matrix [1, 4, 31]. Endothelial proliferation accompanies cell invasion and migration, and lumens of new capillaries are formed when endothelial cells adhere to one another and form tubes. Homeobox genes are master regulatory genes with diverse functions in many cell types, both during embryogenesis and in the adult [10–13]. It is therefore not surprising that recently they have been implicated as important transcriptional regulators controlling endothelial cell phenotype during angiogenesis.

Until recently, little was known about how homeobox genes might influence endothelial cell phenotype and behavior during angiogenesis. However, evidence for their involvement in the phenotypic changes endothelial cells undergo during angiogenesis is now accumulating. For instance, Patel *et al.* reported an endothelial cell-specific variant of *HOXA9* whose expression is regulated by tumor necrosis factor-α, which is proangiogenic [32]. More direct evidence for the importance of homeobox genes in angiogenesis exists for *HOXD3*. Stimulation of endothelial cells with bFGF induces *HOXD3* expression, as well as integrin $\alpha_v\beta_3$ and the urokinase plasminogen activator, effects that are blocked by *HOXD3* antisense. *In vivo,* sustained expression of *HOXD3* on the chick chorioallantoic membrane retains endothelial cells in an invasive state and prevents vessel maturation, leading to vascular malformations and endotheliomas [33]. In diabetic mice, *HOXD3* expression is impaired in endothelial cells, as is its upregulation after wounding [34]. More recently, overexpression of another homeobox gene, HOXB3, in the chick chorioallantoic has been shown to result in an increase in capillary vascular density and angiogenesis, and its blockade by antisense results in impaired capillary morphogenesis [35].

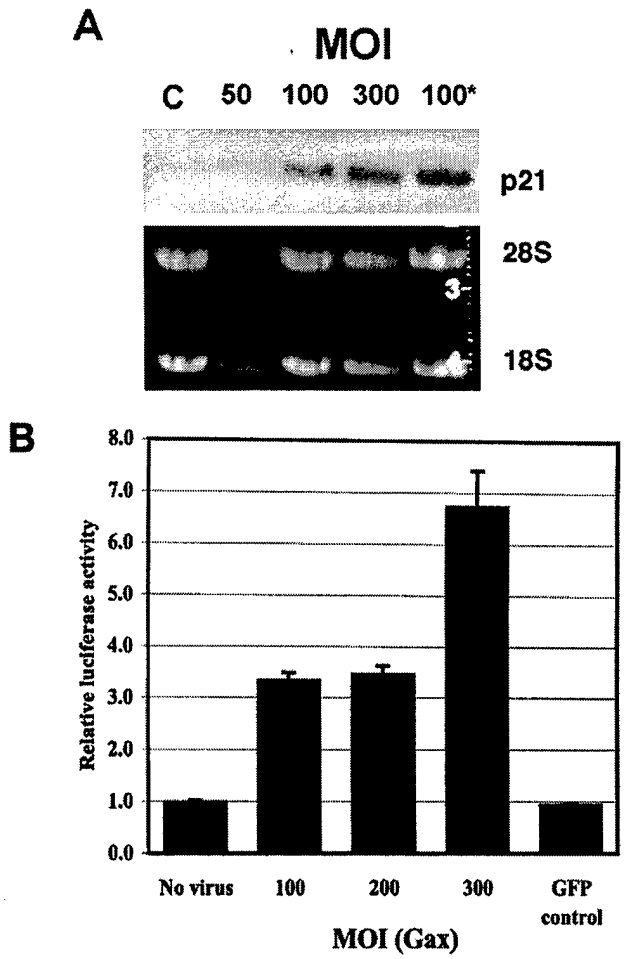

FIG. 5. *Gax* overexpression induces p21 expression. (A) *Gax* expression induces p21 expression in HUVECs. Randomly cycling HUVECs were infected with either Ad.*hGax* at varying MOIs, Ad.*rGax* at MOI = 100(*), or Ad.*GFP* = 300 MOI (C) and then were harvested 24 h later, and Northern blots performed using a p21 probe. (B) Gax expression induces p21 promoter activity. HUVECs were infected with Ad.*rGax* and then transfected with a plasmid containing the p21 promoter driving the firefly Luciferase gene. Luciferase activity was measured 24 h later and normalized to *Renilla* Luciferase activity. Error bars represent standard deviation of 3 wells.

Taken together, these data suggest significant roles for specific homeobox genes in responding to extracellular signals and activating batteries of downstream genes to induce the phenotypic changes in endothelial cells associated with angiogenesis. These observations are what initially led us to look for additional homeobox genes likely to be involved in the final transcriptional control of genes determining angiogenic phenotype.

In this study, we have reported data strongly suggesting a role for another homeobox gene, the growth arrest homeobox gene *Gax,* in regulating the phenotypic changes that occur in vascular endothelial cells during angiogenesis. Moreover, unlike cell cycle regulators such as p21 or p53, the expression of this gene is relatively restricted to the cardiovascular system [14, 15]. We suspected such a role for *Gax* in endothelial cells during angiogenesis because of its activities in vascular smooth muscle cells, which include G_1 cell cycle arrest [18]; p21 activation [18]; and inhibition of migration towards cytokines and mitogens [19]. We therefore looked for its expression in vascular endothelial cells using RT-PCR, Northern blot, and immunohistochemistry and found that *Gax* is indeed expressed in endothelial cells, both *in vitro* (Fig. 1) and *in vivo* in normal human blood vessels (Fig. 2). Moreover, its expression blocks endothelial cell proliferation, with this inhibition being associated with an upregulation of p21. This upregulation is proportional to the level of expression of *Gax,* and appears to be caused by the activation of the p21 promoter.

Tumor angiogenesis represents a promising new target for anticancer therapy. Given that the most important cell in this process is the vascular endothelial cell, targeting angiogenesis implies targeting vascular endothelial cell processes important to angiogenesis. Specific transcription factors such as *Ets-1* [36] are known to integrate the signals coming from the pathways activated by pro- and antiangiogenic factors and translate these signals to changes in endothelial cell gene expression and phenotype. As such, endothelial cell transcription factors represent both a tool for understanding the phenotypic changes endothelial cells undergo in response to proangiogenic factors secreted by tumor cells that result in angiogenesis and potential targets for the anti-angiogenic therapy of cancer. *Gax* is a homeobox transcription factor originally isolated in vascular smooth muscle cells that has previously been shown to be involved in cardiovascular remodeling [19, 21, 37], inhibiting vascular smooth muscle cell proliferation [18] and migration [19]. We have now shown that *Gax* is also expressed in vascular endothelial cells (Figs. 1 and 2). Moreover, *Gax* inhibits endothelial cell proliferation (Figs. 3 and 4) as well, activating p21 expression (Fig. 5). Of most interest, *Gax* also strongly inhibits tube formation on reconstituted basement membranes (Fig. 6), suggesting that, in addition to its role in inhibiting vascular smooth muscle cell-dependent vascular remodeling processes such as intimal hyperplasia [18, 19], it may also have a role inhibiting vascular remodeling processes that depend mainly on endothelial cells, such as angiogenesis. We therefore conclude that *Gax* may represent an important negative regulator of angiogenesis in vascular endothelial cells, and as such may represent a new molecular tool to understand the transcriptional control of changes in gene expression that occur in endothelial cells during angiogenesis and, more importantly, a potential target for the antiangiogenic therapy of cancer.

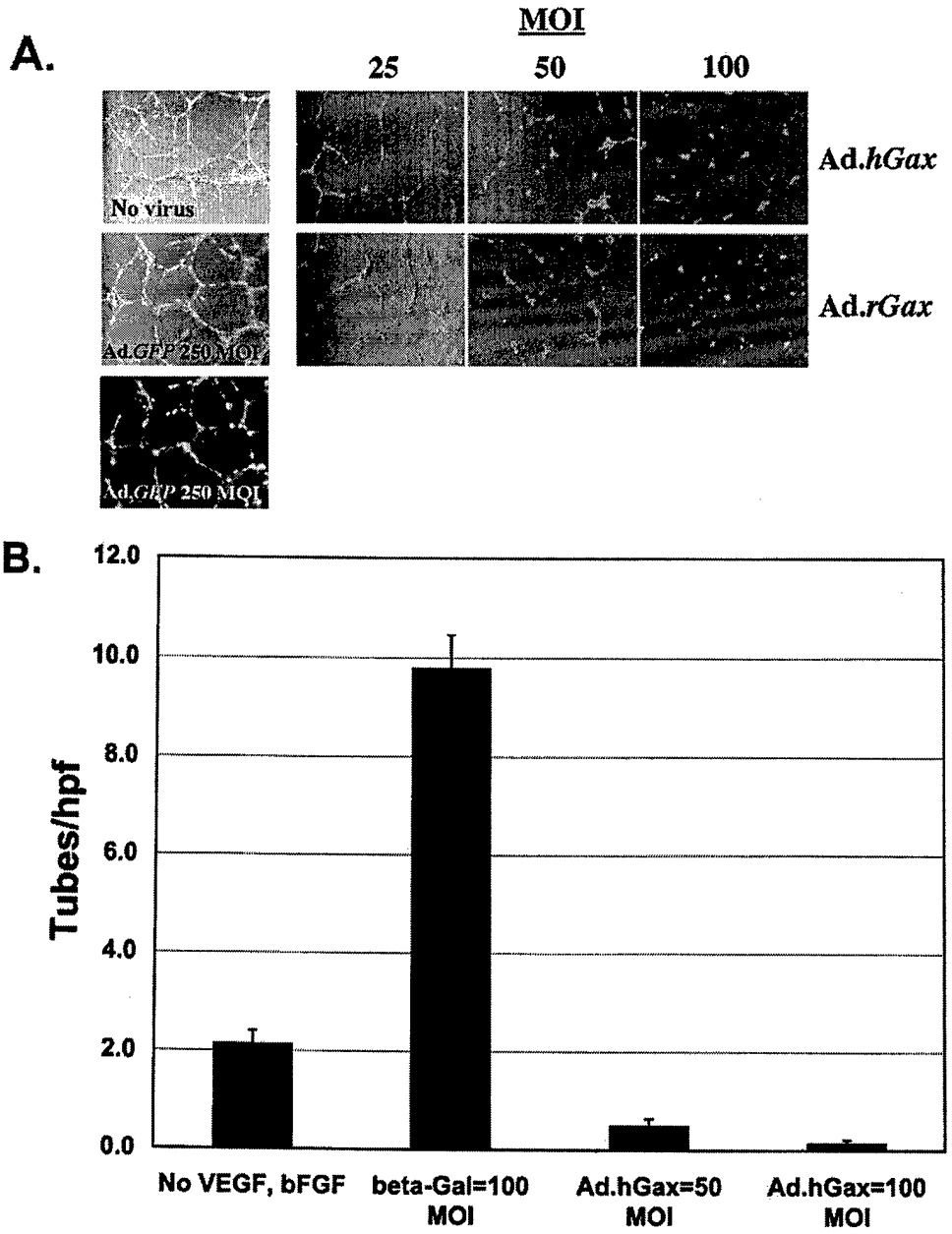

FIG. 6. *Gax* inhibits VEGF-induced endothelial cell tube formation on Matrigel. HUVECs were infected with adenoviruses expressing either human *Gax* (Ad.*hGax*), rat *Gax* (Ad.*rGax*), or *GFP* (Ad.*GFP*) at the MOI indicated. Eighteen hours later, 5×10^5 cells were plated on Matrigel in 6-well plates and incubated overnight in the presence of serum and 10 ng/ml VEGF. Tube formation was strongly inhibited by both Ad.*hGax* and Ad.*rGax* ($P < 0.05$ at MOI = 25). (A) HUVECs in culture demonstrating the inhibition of tube formation by increasing MOI of Ad.*hGax* and Ad.*rGax* and Ad.β-gal was the control. (B) Tube counts for an experiment in which Ad.*hGax* was used to inhibit endothelial cell tube formation.

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ELSEVIER

Control of Vascular Cell Differentiation by Homeobox Transcription Factors

David H. Gorski* and Kenneth Walsh

Homeobox genes are a family of transcription factors with a highly conserved DNA-binding domain that regulate cell proliferation, differentiation, and migration in many cell types in diverse organisms. These properties are responsible for their critical roles in regulating pattern formation and organogenesis during embryogenesis. The cardiovascular system undergoes extensive remodeling during embryogenesis, and cardiovascular remodeling in the adult is associated with normal physiologic processes such as wound healing and the menstrual cycle, and disease states such as atherosclerosis, tumor-induced angiogenesis, and lymphedema. Aside from their roles in the formation of the embryonic vascular system, homeobox genes recently have been implicated in both physiologic and pathologic processes involving vascular remodeling in the adult, such as arterial restenosis after balloon angioplasty, physiologic and tumor-induced angiogenesis, and lymphangiogenesis. Understanding how homeobox genes regulate the phenotype of smooth muscle and endothelium in the vasculature will improve insight into the molecular mechanisms behind vascular cell differentiation and may suggest therapeutic interventions in human disease.
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Changes in cellular phenotype leading to remodeling in the vascular system occur during normal development and in pathologic states. During embryogenesis, vascular endothelial cell (EC) precursors converge into blood islands, which ultimately develop into the aortic arches and capillary networks that provide oxygen and nutrients to the developing organs and limbs. From this, lymphatic EC precursors bud from embryonic veins to form the lymphatic vascular system. In the adult, examples of changes in vascular cell phenotype leading to vascular remodeling include wound healing and the menstrual cycle, during which both angiogenesis and regression of blood vessels are tightly regulated. Examples of pathologic remodeling include atherosclerosis and arterial restenosis after balloon angioplasty. In both processes, vascular smooth muscle cells (VSMCs) migrate from the media to the intima and proliferate, leading to narrowing of the arterial lumen and the subsequent complications, including hypoxia or even anoxia in downstream tissues (Ross 1993)—quickly in the case of restenosis and slowly in the case of atherosclerosis. In addition, phenotypic changes in vascular ECs leading to vascular remodeling play a critical role in tumor biology because diffusion of oxygen and nutrients limits tumor growth to within 1 mm of a capillary. To overcome this limitation, tumors secrete proangiogenic factors to stimulate the ingrowth of new blood vessels (Folkman 1995), which develop from ECs with an immature phenotype (Eberhard et al. 2000). Similarly, tumors also secrete prolymphangiogenic factors, which allow for the ingrowth of lymphatics and subsequent metastasis to regional lymph nodes (Skobe et al. 2001). Thus, understanding the mechanisms underlying the phenotypic changes that lead to vascular remodeling could produce insights into diseases as diverse as atherosclerosis or restenosis, lymphedema, and cancer.

Although the receptors and signaling pathways activated by growth factors and cytokines have been studied extensively in the vascular system, much less is known about the molecular biology of the downstream transcription factors activated by these pathways to regulate tissue-specific gene expression controlling the growth and differentiation of these cells. Transcription factors represent a common mechanism that can integrate multiple signaling pathways to produce the necessary changes in gene expression and phenotype for vascular cells to perform their functions. Homeobox genes encode a family of transcription factors

containing a common 60-amino-acid DNA-binding motif known as the homeodomain, containing a helix–turn–helix motif similar to that found in prokaryotic regulatory proteins such as Cro, CAP, and the λ repressor in *Escherichia coli* (Scott et al. 1989). They are regulators of cell differentiation, proliferation, and migration in both vertebrates and invertebrates, controlling pattern formation in the embryo and organogenesis, as well as oncogenesis in the adult (Cillo et al. 1999, Ford 1998, Krumlauf 1994). Given these characteristics, homeobox genes are excellent candidates for important roles in the final transcriptional regulation of genes responsible for vascular remodeling and angiogenesis in normal physiology and disease. Recently several homeobox genes have been implicated in the phenotypic changes in vascular cells that lead to intimal hyperplasia, arterial restenosis after angioplasty, angiogenesis, and lymphangiogenesis. It is therefore an opportune time to review briefly what is currently known about homeobox gene expression and activity during vasculogenesis and vascular remodeling in the adult.

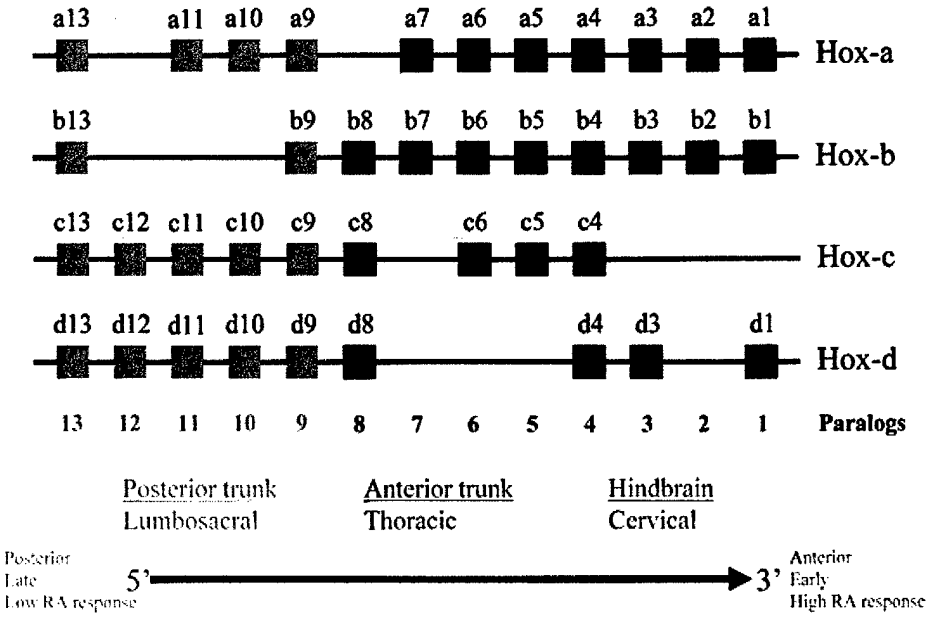

Figure 1. Organization of the HOX clusters. The four HOX clusters in the human and mouse are believed to have evolved through gene duplication. In the human, there are 39 homeobox genes in the HOX clusters (Kosaki et al. 2002). In the mouse, as shown in this figure, the 3′ genes are expressed early in embryogenesis in the more rostral regions of the embryo, whereas the 5′ genes are expressed later in embryogenesis in the caudal regions of the embryo (Cillo et al. 1999). The 3′ rostral genes are highly responsive to retinoic acid (RA), whereas the 5′ caudal genes are less sensitive. Each homeobox gene can have as many as three paralogs in the same position in other HOX clusters. Each HOX cluster is located on a different chromosome. The arrangement of the human HOX clusters, HOX A through D, is nearly identical to the mouse. See text for details.

• Homeobox Gene Expression and Function During Vascular Development

HOX Cluster Genes

In *Drosophilia melanogaster* and vertebrates, many, but not all, homeobox genes are arranged in gene clusters. In mice and humans, there are four unlinked complexes—HOX A through HOX D—that arose from gene duplication (Krumlauf 1994). Because of this, each HOX gene may have as many as three paralogues. The location of each HOX gene in the cluster corresponds to its axial pattern of expression in the developing embryo, with 5′ genes expressed more toward the caudal region and 3′ genes expressed more toward the rostral region (Figure 1), with specific embryonic defects due to knockouts of specific HOX genes occurring in the axial region of their expression. HOX genes have been studied widely with regard to their ability to control pattern formation in the developing embryo. They are powerful regulators of pattern formation, as evidenced by the homeotic mutations (i.e., mutations in which one normal body part is substituted for another normal body part, as in *Antennapedia*).

Several members of the HOX clusters are expressed in the cardiovascular system during embryogenesis, including *HOXA5*, *HOXA11*, *HOXB1*, *HOXB7*, and *HOXC9* (Miano et al. 1996). Moreover, there is functional evidence for involvement of HOX genes in vasculogenesis. For example, transgenic mice with null mutations of the *HOXA3* gene die shortly after birth, suffering from defects in the cardiovascular system that include heart-wall malformations, persistent patent ductus arteriosus, and aortic stenosis (Chisaka and Capecchi 1991). In some of these mice, the right carotid artery fails to form, and in all mice the aorta is thin walled and poorly developed. The overall constellation of defects in *HOXA3* null mice is similar to that observed in the human congenital disorder DiGeorge syndrome (Chisaka and Capecchi 1991).

Because paralogous HOX genes have similar DNA-binding domains and axial expression patterns during embryogenesis, it has been hypothesized that they may have overlapping or complementary functions. Thus, targeting one paralogue may not produce an observable phenotype. This has been demonstrated by antisense targeting of the messages for the paralogous HOX 3 group (*HOXA3* and *HOXB3*), which results in the regression of aortic arch 3 in a manner similar to that of arch 2 (Kirby et al. 1997). Similarly, targeting paralogous group 5 genes (*HOXA5*, *HOXB5*, and *HOXC5*) causes the appearance of an additional pharyngeal arch containing a novel and aortic arch artery (Kirby et al. 1997). These observations suggest that paralogues probably have overlapping functions in vascular development and that in at least some cases they can compensate for each other when the function of one is impaired.

Paired-Related Genes

The expression of two genes not located in the HOX clusters—*Prx1* (formerly known as *MHox* or *Phox*) (Cserjesi et al. 1992) and *Prx2* (formerly known as *S8*) (Opstelten et al. 1991)—during embryogenesis suggests that they have an important role in vasculogenesis. In the vascular

system, expression of *Prx1* and *Prx2* is associated with the primary vessel wall and becomes increasingly restricted to the adventitial and outer medial cell layers as development proceeds (Bergwerff et al. 1998). *Prx1* expression colocalizes with procollagen I and fibrillin 2 but not with smooth muscle α actin, whereas *Prx2* expression is highly associated with the developing ductus arteriosus and is one of the earliest markers of its differentiation. Transgenic mice with null mutations *Prx1* and *Prx2* suggest their relative importance in vascular patterning in the embryo. $Prx2^{-/-}$ mutants do not show cardiovascular malformations. In contrast, $Prx1^{-/-}$ mutants display abnormal positioning and awkward curvature of the aortic arch, in addition to a misdirected and elongated ductus arteriosus (Bergwerff et al. 2000). However, $Prx1^{-/-}/Prx2^{-/-}$ double mutants demonstrate a more severe form of these abnormalities, some of them possessing an anomalous retroesophageal right subclavian artery, as well as excessive tortuosity of all great vessels as they run through the mesenchyme, although they do not have cardiac anomalies (Chesterman et al. 2001). Thus, the loss of *Prx2* function exacerbates anomalies due to the loss of *Prx1*, suggesting functional overlap between these two genes in vascular development.

Hex: *An Early Marker of EC Precursors and Regulator of EC and VSMC Differentiation*

Hex is a proline-rich divergent homeobox gene originally isolated from hematopoietic tissues (Crompton et al. 1992). Expressed in a range of hematopoietic progenitor cells and cell lines (Crompton et al. 1992), *Hex* is an early marker of EC precursors and is transiently expressed in the nascent blood islands of the visceral yolk sac and later in embryonic angioblasts and endocardium (Thomas et al. 1998). The *Xexnopus laevis* homologue *XHex* is expressed in vascular ECs throughout the developing vascular network, and its overexpression leads to disruption of vascular structures and an overall increase in EC number (Newman et al. 1997). These observations suggest an important role for *Hex* in the vascular patterning due to the migration and proliferation of EC precursors. In addition, it has been reported recently that *Hex* also is expressed in VSMCs (Sekiguchi et al. 2001). Its expression is upregulated in neointimal VSMCs after balloon injury in the rat, and *Hex* activates the promoter of NMHC-B/SMemb, a nonmuscle-specific isoform of the smooth muscle myosin heavy chain that is expressed during embryonic development of the aorta, declines in the neonate and adult, and is re-induced in vascular lesions.

Given the above experimental observations, it has been assumed that *Hex* promotes the conversion of ECs to the angiogenic phenotype. However, recent evidence does not support that assumption and suggests that the role of *Hex* in controlling vascular phenotype may be more complex than first thought. First, disruption of the *Hex* gene in mouse embryos does not produce a detectable change in the vascular phenotype (Barbera et al. 2000), suggesting that other factors—perhaps the transcription factor *Scl* (Liao et al. 2000)—may compensate for the loss of *Hex* function. Also, it has been reported recently that *Hex* overexpression in human umbilical vein ECs (HUVECs) inhibits in vitro surrogates for angiogenesis, including migration toward vascular endothelial growth factor (VEGF), invasion, proliferation, and tube formation on reconstituted basement membrane (Matrigel) (Nakagawa et al. 2003). In addition, *Hex* was shown to inhibit the expression of angiogenesis-related membrane genes, including those encoding VEGFR-1, VEGFR-2, neuropilin 1, integrin subunit α_V, Tie-1, and Tie-2. It remains to be clarified whether *Hex* inhibits angiogenesis in vivo, but, taken together with previous reports, these observations suggest a complex role for *Hex* in regulating the proliferation and development of the vascular tree and the differentiation of ECs and VSMCs.

Prox1 *and Development of the Lymphatic System*

The lymphatic system is a vascular network of thin-walled capillaries and larger vessels lined by a layer of ECs that drain lymph from the tissue spaces of most organs and return it to the venous system for recirculation. Early in development, primitive lymph sacs develop from endothelial budding from the veins to form the lymphatic system. The homeobox gene *Prox1* has been implicated in the development of the lymphatic system. Originally isolated by its homology to the *Drosophila* gene *prospero* (Oliver et al. 1993), *Prox1* has an expression pattern that suggests a functional role in a variety of tissues, including eye lens, central nervous system, and liver, with null mutations leading to embryonic lethality (Wigle and Oliver 1999). Supporting a role in lymphatic development is the observation that *Prox1* is the earliest marker of lymphatic EC precursors, and in $Prox1^{-/-}$ knockout mice, budding of ECs that give rise to the lymphatic system is arrested at embryonic day 11.5, resulting in mice without lymphatic vasculature (Wigle and Oliver 1999). In contrast, vasculogenesis and angiogenesis are unaffected by the loss of *Prox1* function (Wigle and Oliver 1999, Wigle et al. 2002). In addition, expression of *Prox1* in vascular ECs results in proliferation and a reprogramming of these cells to a lymphatic EC phenotype, inducing expression of lymphatic genes such as *VEGFR-3*, $p57^{kip2}$, and *desmoplakin I/II* and downregulating vascular EC genes such as *STAT6* and *neuropilin 1* (Hong et al. 2002, Petrova et al. 2002). Moreover, this lymphatic reprogramming due to *Prox1* expression occurs only in vascular ECs, although *Prox1* is still able to induce cyclin expression and proliferation in other cell types (Petrova et al. 2002). Together, these data suggest a role for *Prox1* as a general inducer of proliferation and a key regulatory gene in the developing lymphatic system.

• Homeobox Gene Expression and Function in Mature Blood Vessels

Homeobox Gene Expression during VSMC Phenotypic Modulation and Vascular Disease

VSMCs exist within a spectrum of phenotypes ranging from the "contractile" to the "synthetic" state (Ross 1993). Cells in the contractile state are quiescent; do not migrate; are relatively insensitive to mitogens; express contractile proteins, including smooth muscle-specific isoforms of actin and myosin; and are associated with normal vessel wall. Synthetic state cells, on the other hand, are able to migrate; express lower levels of contractile proteins, with higher levels of nonmuscle isoforms of myosin and actin; secrete extracellular matrix components; and generally resemble less-differentiated VSMCs found in fetal blood vessels. Over the last decade, evidence has been accu-

mulating that homeobox genes are involved in regulating the transition between these two phenotypes.

In the adult, several members of the HOX clusters are expressed in the cardiovascular system. Homeobox sequences isolated from adult rat aorta include *HOXA2*, *HOXA4*, *HOXA5*, and *HOXB7*, and *HOXA11* (Gorski et al. 1994, Patel et al. 1992). Other groups have reported the expression of *HOXA5*, *HOXA11*, *HOXB1*, *HOXB7*, and *HOXC9* in human adult and fetal aortic smooth muscle (Miano et al. 1996, Patel et al. 1992). Of these, *HOXB7* and *HOXC9* are expressed at markedly higher levels in embryonic VSMCs compared with adult VCMCs, suggesting a role in the proliferation and remodeling that occur during embryogenesis (Miano et al. 1996). In addition, overexpression of *HOXB7* in C3H10T1/2 cells results in increased proliferation; the induction of a VSMC-like morphology; and the expression of early, but not intermediate, VSMC markers. Moreover, *HOXB7* mRNA was detected in human atherosclerotic plaques at a higher level than in normal human arterial media (Bostrom et al. 2000). These observations suggest a role for *HOXB7* and perhaps *HOXC9* in vascular remodeling, either in the expansion of immature VSMCs or the change of vascular myocytes to a more immature phenotype, both of which occur in human vascular diseases, such as atherosclerosis and restenosis after balloon angioplasty.

Gax *and Control of Smooth Muscle Phenotype*

Originally isolated from a rat aorta cDNA library with the use of degenerate oligonuceotide probes directed at the most conserved protein sequence of the *Antennapedia* homeodomain (Gorski et al. 1993a), *Gax* (also known as *Mox-2*) encodes a homeodomain-containing transcription factor whose expression has multiple effects on vascular phenotype. Although its expression is more widespread in the embryo, including all three muscle lineages and brain (Skopicki et al. 1997), *Gax* expression in the adult is more narrowly confined to cardiovascular tissues, including heart, medial smooth muscle cells of arteries, lung, and mesangial cells in the kidney (Gorski et al. 1993a). In VSMCs, *Gax* expression is downregulated rapidly by mitogenic signals such as serum, platelet-derived growth factor (Gorski et al. 1993a), and angiotensin II (Yamashita et al. 1997), and more slowly upregulated by growth arrest signals such as serum deprivation (Gorski et al. 1993a) and C-type natriuretic peptide (Yamashita et al. 1997). Moreover, *Gax* expression is also downregulated in the proliferating VSMCs of the rat carotid artery after balloon injury (Weir et al. 1995). *Gax* expression induces G_0/G_1 cell-cycle arrest and upregulates p21 expression by a p53-independent mechanism, and it is this upregulation of p21 that accounts for its antiproliferative activity (Smith et al. 1997). *Gax* also controls the migration of VSMCs toward chemotactic growth factors through its ability to alter integrin expression, downregulating integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ through the specific suppression of the β_3 and β_5 subunits, both in vitro and in vivo (Witzenbichler et al. 1999). Cell-cycle arrest, which does not by itself suppress VSMC migration, is essential for the antimigratory activity of *Gax*, as *Gax* overexpression has no effect on $p21^{-/-}$ cells. Collectively, these data suggest that *Gax* may function to coordinate vascular cell growth and motility through its ability to regulate integrin expression in a cell-cycle-dependent manner. The ability of *Gax* to induce apoptosis in proliferating VSMCs (Perlman et al. 1998) is consistent with these observations, because integrin signaling is an important regulator of cell viability.

Control of Smooth Muscle Phenotype by Prx

The expression of *Prx1* and *Prx2* cannot be detected in the vasculature of adult rats, but they are upregulated in rat pulmonary arteries in which pulmonary hypertension was induced by the injection of monocrotaline (Jones et al. 2001). Induction of *Prx1* and *Prx2* expression in vitro and in vivo is coincident with induction of the extracellular matrix protein tenascin C, which promotes growth and survival of cultured VSMCs. *Prx1* activates the tenascin-C promoter and induces VSMC proliferation in vitro. Consistent with these observations, *Prx1* is upregulated by angiostatin II and, along with the serum response factor, mediates angiotensin II-induced smooth muscle α-actin expression in VSMCs (Hautmann et al. 1997). Collectively, it appears that *Prx1* and *Prx2* genes have roles both in regulating the proliferation of embryonic VSMCs during the formation of the vascular system and in controlling the change of mature VSMCs to a more immature phenotype that occurs in some vascular diseases.

Homeobox Genes and Postnatal Angiogenesis

Functional evidence for the involvement of HOX cluster genes in the regulation of the angiogenic phenotype comes from the study of the paralogous HOX genes *HOXD3* and *HOXB3*, each of which appears to have distinct and complementary roles in this process. *HOXD3* is expressed at high levels in proliferating ECs induced to form tubes on Matrigel but not in quiescent ECs, and its expression is induced by basic fibroblast growth factor (bFGF) (Boudreau et al. 1997). Functionally, blocking *HOXD3* expression with antisense inhibits the bFGF-stimulated upregulation of integrin $\alpha_V\beta_3$ and urokinase plasminogen activator (uPA) without affecting EC proliferation. In contrast, overexpressing *HOXD3* leads to expression of these genes and a morphologic change to the angiogenic phenotype, resulting in the formation of endotheliomas in vivo. In diabetic mice, *HOXD3* expression is impaired in ECs, as is its upregulation after wounding, suggesting that impaired *HOXD3* expression might be involved in the impaired wound healing observed in diabetics (Uyeno et al. 2001). In addition, the *HOXD3* paralogue, *HOXB3*, has been reported to influence angiogenic behavior in a manner distinct from *HOXD3*. Antisense against *HOXB3* impairs the capillary morphogenesis of dermal microvascular ECs and decreases the phosphorylation of the Eph A2 receptor (Myers et al. 2000). Consistent with this result, constitutive expression of *HOXB3* results in an increase in capillary vascular density and angiogenesis, but does not produce endotheliomas. Taken together, these results suggest overlapping and complementary roles for *HOXB3* and *HOXD3* in angiogenesis, with *HOXD3* promoting the invasive or migratory behavior of ECs in response to angiogenic signals and *HOXB3* promoting capillary morphogenesis of these new vascular sprouts.

In contrast to *HOXB3* and *HOXD3*, another HOX cluster gene—*HOXD10*—

inhibits EC conversion to the angiogenic phenotype. Expression of *HOXD10* is higher in quiescent endothelium as compared with tumor-associated vascular endothelium. Moreover, sustained expression of *HOXD10* inhibits EC migration and blocks bFGF- and VEGF-induced angiogenesis in the chick chorioallantoic membrane assay in vivo. Consistent with these observations, human ECs overexpressing *HOXD10* fail to form new blood vessels (Myers et al. 2002) when embedded in Matrigel-containing sponges (Nor et al. 2001) in nude mice. In addition, human ECs overexpressing *HOXD10* express a gene profile consistent with a quiescent, nonangiogenic state, with decreased expression of genes that influence remodeling of the extracellular matrix and cell migration during angiogenesis, such as the uPA receptor and the α_3 and β_4 integrin subunits (Myers et al. 2002). Based on these observations, coupled with the proangiogenic activity of *HOXB3* and *HOXD3*, it has been proposed that the 5′ and 3′ HOX genes have distinct influences on EC behavior, with the more 3′ genes tending to promote the angiogenic phenotype and the more 5′ HOX genes such as *HOXD10* tending to be inhibitory to the angiogenic phenotype and dominant.

The expression of other members of the HOX clusters also have been detected in vascular ECs. One example is *HOXA9EC*, an alternatively spliced variant of HOXA9 whose expression is downregulated by tumor necrosis factor α (TNF-α), which, in addition to its numerous other activities, is proangiogenic (Patel et al. 1999). Also, the expression of several members of the HOX B cluster in HUVECs is regulated by VEGF and tissue plasminogen activator, but not bFGF (Belotti et al. 1998). Because HOX B cluster gene expression does not correlate with the mitogenic state of the cell but rather is altered with the state of cellular differentiation, it has been suggested that these genes are involved in the morphogenic changes associated with the angiogenic phenotype.

Recently it has been reported that *Gax* also is expressed in vascular ECs (Gorski and Leal 2003). As in VSMCs, in ECs, *Gax* expression results in cell-cycle arrest and induces p21 expression and promoter activity. Of note, it also strongly inhibits EC tube formation in response to VEGF on Matrigel (Gorski and Leal 2003) in a manner similar to that of *Hex* (Nakagawa et al. 2003). These additional observations suggest that in addition to its likely role in maintaining VSMCs in the contractile phenotype, *Gax* may also have a role in EC differentiation. Taken together, all of the above observations suggest that *Gax* may be a global inhibitor of vascular cell activation. However, like *Hex* knockout mice (Barbera et al. 2000), mice transgenic for a null mutation in *Gax* have not been reported to show vascular anomalies (Mankoo et al. 1999). Rather, they show skeletal muscle anomalies in the limbs and die shortly after birth from unknown causes. This would tend to suggest that other homeobox factors, such as *Mox-1* (Candia and Wright 1996) or possibly *Pax3* (Stamataki et al. 2001), might compensate for a lack of *Gax/Mox-2* expression in the developing cardiovascular system. It would be of great interest to determine whether *Gax* knockout mice demonstrate increased angiogenesis in response to proangiogenic stimuli, but such studies would be difficult because of their very brief life span. Similar studies would also be of interest in *Hex* knockout mice.

Other homeobox genes also are likely to be involved in regulating angiogenesis, whether physiologic or tumor induced. For example, St. Croix et al. (2000) used serial analysis of gene expression to look for expressed sequence tags (ESTs) whose expression is at least 10-fold greater in tumor endothelium compared with normal endothelium. Not surprisingly, many of the ESTs they reported derive from extracellular matrix proteins. However, one EST was similar to the homeobox gene *Dlx-3*, a member of the *Distal-less* family of homeobox genes. This EST was not detectable in the developing corpus luteum, implying a distinction between tumor angiogenesis and physiologic angiogenesis. Interestingly, *Dlx-3* has been implicated in placental function (Beanan and Sargent 2000). Other placental homeobox genes include *Dlx-4*, *Gax/Mox-2*, *HB24*, and *Msx2* (Quinn et al. 1997). Given the critical importance of angiogenesis and blood vessel regression in placental function, it is reasonable to predict that some of these genes are involved in vascular remodeling in the placenta. It is also reasonable to postulate that homeobox genes previously demonstrated to be important in inducing proliferation and migration of ECs and EC precursors during angiogenesis—such as *Hex*—also may be important in inducing angiogenesis in the adult vasculature.

• Conclusions

Although much more is known since the last time we reviewed the expression and function of homeobox genes in the vasculature (Gorski et al. 1993b), knowledge of the transcriptional regulation of VSMC and EC phenotype still is not as detailed as is the understanding of the cytokines and growth factors that act on ECs and VSMCs to regulate their phenotype, the receptors these factors activate, and the downstream signaling pathways activated in turn by these receptors. However, a growing number of homeobox genes have been implicated in vascular development in the embryo and vascular remodeling, angiogenesis, and vascular diseases in the adult. Moreover, with the description of *Prox1* (Hong et al. 2002, Petrova et al. 2002), it has become clear that homeobox genes participate in the development of the lymphatic vascular system as well. Given the sheer number of homeobox genes and potential interactions between them and vascular remodeling, it is difficult to generalize too much about the roles of homeobox genes in these processes, some of which are listed in Table 1. It is possible, however, to come to three general conclusions with regard to how homeobox genes regulate vascular remodeling.

1. Pathways controlled by homeobox genes are redundant, especially during embryogenesis. This implies that it is more likely to be the overall pattern of homeobox gene expression rather than any one individual homeobox gene that regulates the phenotype of VSMCs and ECs during angiogenesis and vascular remodeling. The roles of *HOXB3*, *HOXD3*, and *HOXD10* in regulating EC phenotype during angiogenesis represent a good example of this principle. It may be the balance between pro- and antiangiogenic HOX cluster genes that determine whether an EC becomes angiogenic, and different proangiogenic HOX genes may control different stages or aspects of angiogenesis (e.g., *HOXB3* and *HOXD3*). It also can be postulated that *Gax* and *Hex* help to determine this balance. Similarly, in VSMCs, it can be postulated that the balance between *Gax* and *Prx1/Prx2* (and possibly *Hex*) plays a major role in

Table 1. Homeobox genes expressed in the cardiovascular system

Cell type	*Gene*	*Function/observation*	*Reference*
VSMC	*Gax (Mox-2)*	Downregulated upon mitogen stimulation and vascular injury Causes G_1 cell-cycle arrest and inhibits VSMC migration Inhibits integrin $\alpha_V\beta_3$ and $\alpha_V\beta_5$ expression Induces apoptosis in cycling cells Inhibits restenosis after balloon injury Interacts with *Pax3*	Gorski et al. 1993a, Perlman et al. 1998, Smith et al. 1997, Stamataki et al. 2001, Weir et al. 1995, Witzenbichler et al. 1999, Yamashita et al. 1997
	Hex	Induces expression of immature actin isoform in VSMCs	Sekiguchi et al. 2001
	HOX B7	More highly expressed in fetal VSMCs than in adult VSMCs Induces differentiation of C3H10T1/2 cells into VSMC-like cells	Bostrom et al. 2000, Miano et al. 1996
	HOX C9	More highly expressed in fetal VSMCs than in adult VSMCs	Miano et al. 1996
	HOX A3 and *B3*	*HOX A3* knockout mice have vascular anomalies Blocking *HOX A3* and *B3* causes regression of aortic arch 3	Kirby et al. 1997
	HOX A5, *B5*, and *C5*	Blocking expression causes appearance of additional aortic arch artery	Kirby et al. 1997
	HOX A2, *A4*, *A11*, and *B1*	Isolated from vascular smooth muscle, functions in VSMC unknown	Gorski et al. 1993a and 1994, Patel et al. 1992
	Prx1	Interacts with serum response factor to activate binding Putative role in angiotensin II-mediated smooth-muscle α-actin expression *Prx1*/*Prx2* double-null mutants demonstrate vascular anomalies Activates proliferation and tenascin-C expression	Bergwerff et al. 1998 and 2000, Chesterman et al. 2001, Hautmann et al. 1997, Jones et al. 2001
	Prx2	Widely expressed in embryonic vasculature *Prx1*/*Prx2* double-null mutants demonstrate vascular anomalies	Bergwerff et al. 1998 and 2000, ten Berge et al. 1998
Vascular ECs	*HOXA9EC*	EC specific, function presently unknown Expression modulated by tumor necrosis factor α	Patel et al. 1999
	HOX B cluster	HOX B cluster induced by differentiating factors	Belotti et al. 1998
	HOXB3	Involved in regulating capillary morphogenesis	Myers et al. 2000
	HOXD3	Induces expression of integrin $\alpha_V\beta_3$ Induces angiogenic phenotype in ECs Impaired function associated with impaired wound healing	Boudreau et al. 1997, Uyeno et al. 2001
	HOXD10	Inhibits angiogenesis and changes EC gene expression profile to the nonangiogenic state	Myers et al. 2002
	Dlx-3	Expressed sequence tags with homology to *Dlx-3* expressed at high levels in tumor endothelium Necessary for placental development	Quinn et al. 1997, St. Croix et al. 2000
	Gax (Mox-2)	Inhibits in vitro surrogates for angiogenesis May have function in placental–mesenchymal interactions	Gorski and Leal 2003, Quinn et al. 1997 and 2000
	Hex	Early marker of ECs during embryogenesis Expressed throughout the vascular network Overexpression increases EC number in embryos Overexpression blocks EC tube formation on Matrigel	Barbera et al. 2000, Liao et al. 2000, Nakagawa et al. 2003, Newman et al. 1997, Sekiguchi et al. 2001, Thomas et al. 1998
Lymphatic ECs	*Prox1*	Specific to lymphatic ECs Induces expression of lymphatic EC-specific genes Null mutations prevent development of lymphatic system Master regulator of lymphatic vessel formation from embryonic venous system	Hong et al. 2002, Petrova et al. 2002, Wigle and Oliver 1999, Wigle et al. 1999 and 2002

EC, endothelial cell; VSMC, vascular smooth muscle cell.

determining whether VSMCs become contractile or synthetic.

2. Individual homeobox genes may function as master regulatory genes for parts of the vascular system. For instance, although a master regulatory gene controlling development of angioblasts into vascular ECs or VSMCs remains to be identified, *Prox1* represents a very good candidate for such a role in lymphatic endothelium. However, it must be remembered that most homeobox genes controlling vascular phenotype also are expressed in other tissues. Even *Prox1* is expressed in liver and eye lens during embryogenesis. Similarly, *Prx1* is clearly important in skeletal development (ten Berge et al. 1998), and *Gax* is important in skeletal muscle development (Mankoo et al. 1999). This implies that cell-type-specific factors influence the activities of homeobox genes in both ECs and VSMCs and that homeobox genes may be downstream from other, more global, master regulatory genes. Indeed, *Prox1* can only reprogram a vascular EC to take on the phenotype of lymphatic endothelium (Petrova et al. 2002). It cannot so reprogram other cell types.

3. Little is known about how homeobox genes implicated in angiogenesis and vascular remodeling exert their effects at the molecular level. However, it is clear that at least a subset of them appear to function by controlling the differentiation, proliferation, and/or migration of VSMCs and ECs. The mechanism behind these phenotypic changes must be the activation and repression of specific batteries of downstream genes. Because few downstream genes from homeobox genes are known, one of the most fertile areas of research for homeobox gene research is the identification of their downstream targets and the elucidation of the mechanisms by which homeobox genes regulate the expression of these target genes and these target genes in turn lead to the phenotypic changes observed. In the near future, it is likely that cDNA microarray technology will provide an excellent tool for identifying the global changes in gene expression occurring in response to homeobox gene expression in vascular cells.

Given their importance in cell-cycle control, cell migration, and cell adhesion, it is likely that many more homeobox genes will be implicated in the regulation of vascular remodeling and angiogenesis. The identification of the specific homeobox genes involved in these processes, their downstream target genes, and the cell-signaling pathways activated and repressed by homeobox gene expression in vascular ECs and VSMCs will result in a better understanding of the basic cellular mechanisms by which the vascular system is remodeled in response to physiologic signals, tumors, or other stimuli. Such understanding has the potential to lead to the development of therapies that block tumor angiogenesis and lymphatic metastasis, reverse atherosclerosis, prevent restenosis after angioplasty, improve wound healing, and reverse lymphedema.

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The homeobox gene *Gax* inhibits endothelial cell activation, angiogenesis, and nuclear factor κB activity

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ABSTRACT

The growth and metastasis of tumors are heavily dependent on angiogenesis, but much of the transcriptional regulation of vascular endothelial cell (EC) gene expression responsible for angiogenesis remains to be elucidated. The homeobox gene *Gax* is expressed in vascular ECs and inhibits proliferation and tube formation *in vitro*. We hypothesized that *Gax* is a negative transcriptional regulator of the EC angiogenic phenotype and studied its regulation and activity in vascular ECs. Several pro-angiogenic factors caused a rapid downregulation of *Gax* mRNA in human umbilical vein endothelial cells (HUVECs), as did conditioned media from breast cancer cell lines. In addition, *Gax* expression using an replication-deficient adenoviral vector inhibited HUVEC migration towards proangiogenic factors *in vitro* and inhibited angiogenesis *in vivo* in Matrigel plugs. To identify potential downstream targets of *Gax*, we examined changes in global gene expression due to *Gax* activity using cDNA microarrays. *Gax* expression resulted in changes in HUVEC gene expression profiles consistent with a quiescent, non-angiogenic phenotype, with increased expression of cyclin kinase inhibitors and decreased expression of numerous genes implicated in EC activation and angiogenesis. Further analysis revealed that *Gax* downregulated numerous nuclear factor-κB (NF-κB) targets genes, and we observed that *Gax* decreases the binding of NF-κB to its target sequence in electrophoretic mobility shift assays. To our knowledge, *Gax* is the first homeobox gene described that influences NF-κB activity in vascular ECs. Because NF-κB has been implicated in EC activation and angiogenesis, the downregulation of NF-κB-dependent genes by *Gax* suggests one potential mechanism by which *Gax* inhibits the angiogenic phenotype.

INTRODUCTION

The process of angiogenesis, critical in both normal physiology and in disease states such as cancer and inflammatory diseases, is normally tightly regulated by a balance between pro- and antiangiogenic factors, known as the "angiogenic balance" (32). Tumors hijack the process of angiogenesis in order to parasitize their host by secreting proangiogenic peptides, cytokines, and chemokines, tipping the "angiogenic balance" towards a proangiogenic state. The primary target of proangiogenic factors secreted by tumors is the vascular endothelial cell (EC). During angiogenesis, whether physiologic or tumor-induced, vascular ECs become "activated" and undergo distinct changes in phenotype and gene expression. These changes include activation of proteolytic enzymes to degrade basement membrane, EC sprouting, proliferation, tube formation, and production of extracellular matrix (3, 45, 60). Although the EC receptors and signaling pathways activated by proangiogenic factors, such as vascular endothelial growth factor (VEGF) (15, 16) or basic fibroblast growth factor (bFGF) (15), and proangiogenic and proinflammatory factors such as tumor necrosis factor-α (TNF-α) (64) have been extensively studied, less is known about the molecular biology of the downstream transcription factors activated by these factors, which in turn activate and repress batteries of downstream genes necessary for the phenotypic changes that ECs must undergo in order for angiogenesis to occur. Nuclear transcription factors likely integrate these signals in order to produce the gene expression profile that results in the phenotypic changes resulting in angiogenesis. Thus, understanding the transcriptional mechanisms by which ECs become activated is likely to suggest new therapeutic strategies for inhibiting this process at a very distal point in its signaling cascade, with potential applications in the treatment of cancer and vascular diseases whose pathophysiology is significantly based on EC activation, such as atherosclerosis.

Because of their ubiquitous role as regulators of cellular differentiation and body plan formation during embryogenesis, as well as oncogenes and tumor suppressors, in various human cancers (1, 13), it is not surprising that homeobox genes have been implicated in regulating the phenotypic changes that ECs undergo when during angiogenesis (4-6, 25, 47-49, 51). In particular, one diverged homeobox genes, *Gax* (**G**rowth **A**rrest-specific homeobo**X**, whose mouse homologue is known as *Mox-2*), has several characteristics that implicate it as potentially having an important role as an inhibitor of the EC phenotypic changes that occur in response to stimulation by proangiogenic or proinflammatory factors (10, 27, 54, 59, 66, 67). Originally isolated from vascular smooth muscle (27) and widely expressed in mesoderm and muscle precursors in the embryo (9, 25, 58), in the adult *Gax* expression is mostly restricted to the cardiovascular system and kidney (27, 58). In vascular smooth muscle cells, *Gax* expression is downregulated by mitogens and upregulated by growth arrest signals (27, 66, 68). Consistent with this observation, *Gax* expression induces G_1 cell cycle arrest (59) and inhibits vascular smooth muscle cell migration, modulating expression integrin expression (67). *In vivo*, *Gax* expression in arteries inhibits proliferative restenosis of the arterial lumen after injury (42, 54, 59). Recently, we have observed that *Gax* has similar effects on EC phenotype, such as inhibition of proliferation associated with the induction of p21 expression (25). In addition, *Gax* strongly inhibits VEGF-induced EC tube formation on reconstituted basement membrane (25), suggesting that *Gax* may be a negative regulator of the phenotypic changes that ECs undergo when they become activated or angiogenic.

Until now, we had not identified potential mechanisms by which *Gax* might accomplish its inhibition of EC activation, other than a general cell cycle arrest due to induction of p21 (25, 59). In this report, we now describe how *Gax* expression is regulated in ECs by proangiogenic

and proinflammatory factors and how its expression can block EC activation *in vitro* and angiogenesis *in vitro* and *in vivo*. Finally, we provide evidence that *Gax* inhibits nuclear factor-κB (NF-κB) signaling in ECs. Given that there is now considerable evidence that activation of NF-κB activity in ECs is proangiogenic (39, 40, 43, 46, 52, 56, 57), this interaction likely represents one potential mechanism by which *Gax* expression may inhibit angiogenesis,. This interaction, to our knowledge the first described in ECs, may represent a new mechanism by which homeobox genes can interact with intracellular signaling pathways and thereby inhibit angiogenesis.

METHODS

Cell culture and expression constructs

Human umbilical vein endothelial cells (HUVECs) were obtained from BioWhittaker (Walkersville, MD) and cultured according to the manufacturer's instructions in EGM-2 medium, also obtained from BioWhittaker. HMEC-1 cells were obtained from the Centers for Disease Control and were cultured as described (2).

The construction of replication-deficient adenoviral vectors expressing the rat and human homologs of *Gax* (Ad.*hGax* and Ad.*rGax*, respectively) conjugated to the α-hemagluttinin (HA) epitope has been described previously (59). In this study, both human and rat isoforms of *Gax* were used, in order to verify that both isoforms have similar activity. The control replication-deficient adenoviral vector expressing green fluorescent protein (Ad.*GFP*) was a kind gift of Dr. Daniel Medina (The Cancer Institute of New Jersey, New Brunswick, NJ). As a positive control for inhibition of angiogenesis *in vivo* by a viral vector, we utilized an additional adenoviral construct expressing a form of Akt (T308A, S473A, adenoviral construct designated Ad.DN.Akt) that functions in a dominant negative fashion (22, 50), kindly provided by Dr.

Kenneth Walsh (Boston University). Viral titers were measured by plaque assay. Expression of *Gax* mRNA and protein in cells transduced with these adenoviral constructs were verified by quantitative real time PCR according to methods described below and Western blot utilizing anti-HA antibody (not shown).

Quantitative reverse transcriptase real time polymerase chain reaction (QRT-PCR)

After treatment as described individually for each experiment, total RNA was isolated from ECs using a spin-column with on-column DNase digestion to remove contaminating genomic DNA (RNAeasy, Qiagen). First strand synthesis was performed on the total RNA also using oligo-dT primers (Superscript kit, Invitrogen). Message levels for *Gax* and the endothelial cell adhesion molecules (E-selectin, VCAM-1, and ICAM-1) were then determined by quantitative real time RT-PCR utilizing TaqMan probes (8). QRT-PCR was carried out using a Cepheid SmartCycler thermocycler, with the associated SmartCycler v.2.0 software used to analyze the data and determine the threshold count (C_t).

Primer and probe sets for each gene were designed using the MacVector 7.2 software package (Accelrys, San Diego, CA). The fluorophore used was 6-FAM and Black Hole Quencher-1 (BHQ-1, Biosearch Technologies, Novato, CA). Sequences of the primers and probes were: *Gax*: 5'-TCA GAA GTC AAC AGC AAA CCC AG-3' (forward), 5'-CCA GTT CCT TTT CCC GAG-3' (reverse), 5'-(6-FAM)-TGG TTC CAA AAC AGG CGG ATG-3'-(BHQ1) (TaqMan probe), amplicon = 238 bp; E-Selectin: 5'-CTC TGA CAG AAG AAG CCA AG3' (forward), 5'-ACT TGA GTC CAC TGA AGT CA -3' (reverse), 5'-(6-FAM)-CCA CGC AGT CCT CAT CTT TTT G-3'-(BHQ1) (TaqMan probe), amplicon = 255 bp; VCAM-1: 5'-ATG ACA TGC TTG AGC CAG G -3' (forward), 5'-GTG TCT CCT TCT TTG ACA CT-3' (reverse), 5'-(6-FAM)-CAC TTC CTT TCT GCT TCT TCC AGC-3'-(BHQ1) (TaqMan probe),

amplicon = 260 bp; ICAM-1: 5'-TAT GGC AAC GAC TCC TTC T-3' (forward), 5'-CAT TCA GCG TCA CCT TGG -3' (reverse), 5'-(6-FAM)-CCT TCT GAG ACC TCT GGC TTC G-3'-(BHQ1) (TaqMan probe), amplicon = 238 bp. To correct for differences in RNA quality and quantity between samples, the target gene was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) message levels. Sequences of the GAPDH primer and probe set were: 5'-ACA ACT TTG GTA TCG TGG AAG-3' (forward); 5'-CAG ATG AGG CAG GGA TGA TGT TC -3' (reverse); 5'-(6-FAM)-ACC CAG AAG ACT GTG GAT GG-3'-(BHQ1) (TaqMan probe), amplicon = 138 bp. For some experiments (Figure 1), a set of primers for human *Gax* previously described were used (25).

Reaction mixtures for QRT-PCR were 25 µl, containing 0.75 U *Taq* polymerase (Invitrogen), reaction buffer, 0.2 mM dNTPs, plus optimized concentrations of $MgCl_2$, probe, and primers. For all reactions, negative controls were run with no template present, and random RNA preparations were also subjected to sham QRT-PCR (no reverse transcriptase) to verify lack of contamination with genomic DNA. The PCR cycle started with an initial 1.5 minute denaturation step at 95° C, followed by 30 to 40 cycles of denaturation at 95° C for 10 seconds; annealing at 50° (VCAM-1), 52° (E-selectin, ICAM-1), 56° (*Gax*, GAPDH) for 20 seconds; and extension at 72° C for 30 seconds. Each sample was run in triplicate and C_t determined for the target gene. Target gene mRNA levels ere estimated and normalized to GAPDH mRNA levels using the $\Delta\Delta C_t$ method, as we have done previously (26, 31).

Migration assays

Prior to the experiment cell culture membranes and flasks were coated with sterile 0.1% gelatin in PBS. HUVECs were infected with adenoviral vectors at MOI=100 for 16 hours before 5×10^4 cells/well were plated onto 8.0 µm pore size polycarbonate membrane in 24 well plates.

Cells were allowed to attach for one hour in EGM-2 medium. Once the cells had attached, the medium in the upper chamber was replaced with low serum medium (LSM, which consisted of EGM-2 + 0.1% FBS lacking VEGF, bFGF, and EGF), and the lower chamber with LSM supplemented with either 50 ng/ml VEGF, 50 ng/ml bFGF, 15 ng/ml TNF (VEGF, bFGF, and TNF-α all obtained from R&D Systems, Minneapolis, MN), or 10% FBS. After 5 hours the inserts were washed with PBS and the upper surfaces cleaned with a cotton swab to remove any cells that had not migrated. Finally the cells were fixed with Diff-Quik® Stain (Dade Behring, Deerfield, IL) and the inserts washed in PBS and photographed for counting. Cells were counted in five high powered fields (hpf) per well. Experiments were repeated at least three times.

In vivo angiogenesis assay

The formation of new blood vessels *in vivo* was assayed by the Matrigel plug assay as described previously (36, 50). These experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee at UMDNJ-Robert Wood Johnson Medical School, and this research has complied with all relevant federal guidelines and institutional policies. In brief, cold Low Growth Factor Matrigel (BD Pharmingen, San Diego, CA, 500 μl/mouse) containing bFGF 400 ng/ml (R & D Systems, Minneapolis, MN), heparin 10 U/ml (Sigma, St. Louis, MO), and 10^8 pfu of either Ad.GFP, Ad.*hGax*, or Ad.*rGax* was injected subcutaneously in C57BL/6 mice (N=8 per experimental group). As a positive control for angiogenesis inhibition by a viral vector, we utilized an adenoviral construct expressing a dominant negative form of Akt (Ad.DN.Akt) provided by Dr. Kenneth Walsh (see above), which has previously been used to show that inhibition of Akt signaling inhibits angiogenesis *in vivo* (50). As another control, to verify that adenovirus itself does not significantly alter *in vivo* angiogenesis as measured by this assay, plugs containing only bFGF were also examined.

After 14 days, the mice were euthanized by CO_2 inhalation followed by cervical dislocation, and the plugs carefully removed *en bloc* with surrounding connective tissue. Tissue and plugs were fixed for 10 minutes in cold acetone and frozen sections made. Tissue sections were cut at 5 μm and their endogenous peroxidase activity blocked with dilute H_2O_2. Sections were then blocked with 5% bovine serum albumin (BSA) for 15 minutes, washed with PBS, and then incubated with rat anti-mouse CD31 (PECAM) monoclonal antibody (BD PharMingen, San Diego, CA) at a 1:20 dilution in 1%BSA in PBS) overnight. Sections were then washed with cold PBS twice and incubated with Biotinylated mouse anti-rat $IgG_{1/2a}$ (BD PharMingen, San Diego, CA) in 1% BSA/PBS at 1:400 dilution. Color was then developed with streptavidin-peroxidase (VectaStain, ABC Kit, Vector Laboratories, Burlingame, CA). Sections were counterstained with Toluidine Blue and vessel counts performed as previously described (44, 50). In brief, vascular hot spots were located for each plug near the interface between the plug and surrounding stroma, and blood vessel density estimated as the number of vessels per high-powered field. The fields chosen for each plug section were all located at the plug-stroma interface. Two sections from each plug were made and at least five high-powered fields per section were counted, and the mean ± standard error of the mean determined for each experimental group. The experiment was repeated twice. Statistical differences were determined by one-way ANOVA using Prism v.4.0 (GraphPad Software, Inc., San Diego, CA), followed by Dunnett's multiple comparison test using the Ad.GFP group as the control.

cDNA microarray studies

We compared global gene expression in control HUVECs transduced with Ad.GFP with that of HUVECs transduced with Ad.r*Gax* or Ad.h*Gax*. Cells were transduced at an MOI=100, incubated 24 hours in normal media, then harvested for total RNA isolation as described above

for quantitative real time RT-PCR. RNA quality was verified by electrophoresis through formaldehyde-containing agarose gels prior to use for generating probes for cDNA microarray analysis. Exogenous *Gax* expression was verified by real time quantitative RT-PCR and Western blot (data not shown). Global gene expression was then compared in two separate experiments using the Affymetrix Human Genome U133A GeneChip® array set and standard protocols supplied by the manufacturer, with technical assistance from the cDNA Microarray Core Facility of The Cancer Institute of New Jersey. This chip contains probe sets for over 33,000 known genes, along with probes for housekeeping genes for normalization and genomic DNA for evaluation of hybridization quality. Results were further analyzed with GeneMAPP (19) to identify signal-dependent changes in gene expression.

Western blots

Whole cell extracts from TNF-α treated HUVECs were electrophoresed on 8% SDS-polyacrylamide gels and transferred to polyvinylidene diflouride membranes. The membranes were placed in blocking solution (PBS containing 5% non-fat dry milk and 0.1% Tween-20) for 1 hour or overnight at 4° C before being incubated with the appropriate dilution of primary antibody (mouse monoclonal anti-VCAM-1 and anti-ICAM-1 and rabbit polyclonal anti-E-selectin; Santa Cruz Biotechnology, Santa Cruz, CA) in blocking solution for 1-2 hours. Blots were then washed 3 times with blocking solution and incubated for 1 hour in secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG; Pierce Biotechnology, Inc., Rockford, IL) at a dilution of 1:5000 before being washed 2 times with blocking. A final wash of PBS with 0.1% Tween-20 for 5 minutes was done prior to visualization by chemiluminescence using the ECL-Plus reagent (Amersham, Piscataway, NJ).

Flow cytometry

Cells were harvested after the relevant treatment and resuspended in phosphate buffered saline (PBS) containing 0.1% sodium azide. Approximately 1 x 10^5 cells were incubated with FITC-conjugated primary antibody against human E-selectin, VCAM-1, or ICAM-1 (BD Biosciences, San Diego, CA) for 30 minutes on ice. Cells were pelleted and washed twice in PBS/Azide prior to flow analysis on a Beckman-Coulter Cytomics FC500 flow cytometer (Fullerton, CA).

Electrophoretic mobility shift assays

HUVEC cells were transduced with Ad.GFP or Ad.r*Gax* and then induced with 10 ng/ml TNF-α for 1 hour. Nuclear extracts were prepared with the NE-PER nuclear extraction reagent (Pierce Biotechnology, Rockford IL). Biotin end-labeled double-stranded oligonucleotides containing the NF-κB consensus sequence, 5'-biotin-AGT TGA GGG GAC TTT CCC AGG C-3' were purchased from IDT technologies - Idaho. The binding reactions contained 6-8 μg of nuclear extract protein, buffer (10 mM Tris, pH 7.5, 50 mM KCl, 1 mM dithiothreitol), 1 μg of poly(dI-dC), 5μg BSA and 20 fM of biotin-labeled DNA. The reactions were incubated at room temperature for 20 min. The competition reactions were performed by adding up to 200-fold excess unlabeled double-stranded NF-κB consensus oligonucleotide to the reaction mixture. Other controls included competition with random oligonucleotide (5'-TAG CAT ATG CTA-3') and an NF-κB site with a point mutation that abolishes DNA binding (5'CAC AGT TGA GGC CAC TTT CCC AGG C-3'). The reactions were electrophoresed on a 6% acrylamide gel at 100 V for 1 h in 0.5X Tris-borate-EDTA buffer. The reactions were transferred to positively charged nylon membrane. Biotinylated oligonucleotides were detected with streptavidin linked horseradish peroxidase and the Pierce LightShift kit (Pierce Biotechnology, Rockford, IL).

RESULTS

Gax expression is rapidly downregulated by mitogens and proangiogenic factors in ECs

Given our previous observations that *Gax* expression inhibits EC proliferation and tube formation *in vitro* (25), we first wished to determine how *Gax* expression is regulated by growth factors and proangiogenic peptides in ECs. Because endogenous *Gax* message is usually expressed at relatively low levels in VSMCs and ECs (25, 27), we developed a quantitative real time PCR assay using *Gax*-specific primers and a TaqMan probe (33) (see Methods). First, we studied the time course of *Gax* downregulation. HUVECs made quiescent by incubation for 24 hrs in 0.1% FBS were stimulated with 10% FBS plus 5 ng/ml VEGF. *Gax* was rapidly downregulated by 5-fold within four hours and slowly returned to basal over 24 to 48 hours (Figure 1, A and C). Conversely, when sparsely plated randomly cycling HUVECs were placed in medium containing 0.1% serum, *Gax* was upregulated nearly 10-fold within 24 hours (Figure 1B). We then stimulated quiescent HUVECs with proangiogenic or proinflammatory factors, including bFGF, VEGF, and TNF-α. *Gax* was rapidly downregulated with a similar time course (Figure 2). Similar results were observed in HMEC-1 cells (2), an immortalized human microvascular endothelial cell line that retains many characteristics of microvascular endothelial cells (data not shown).

Next, we compared the efficacy of serum and different proangiogenic factors at downregulating *Gax* expression in quiescent HUVECs (Figure 3). At 4 hours, serum and all growth factors tested downregulated *Gax* similarly (Figure 2 and Figure 3, A and B). However, at 24 hours, serum, VEGF, and TNF-α tend to be more potent at downregulating *Gax* than bFGF (Figure 2 and Figure 3, C and D), although this may be because bFGF requires a higher concentration than VEGF (Figure 3D). Finally, if *Gax* downregulation is a necessary molecular

event that allows ECs to become activated by tumor-secreted proangiogenic factors, we hypothesized that tumor-secreted factors would also downregulate *Gax* expression *in vitro* and *in vivo*. We therefore incubated breast cancer cell lines in serum-free medium for 24 hours, harvested the conditioned medium, and stimulated quiescent HUVECs with the media for 4 hours. The cell lines varied considerably in their ability to downregulate *Gax*, but all of them downregulated *Gax* expression at least three-fold, and some by as much as 20-fold (Figure 4), suggesting that tumor-secreted proangiogenic factors also regulate *Gax* expression.

Gax expression inhibits endothelial cell migration towards proangiogenic factors

Migration of ECs through the basement membrane and into the surrounding stroma in response to proangiogenic stimuli is a critical step in tumor-induced angiogenesis. We therefore tested the ability of *Gax* to inhibit EC migration towards proangiogenic factors. HUVECs were transduced with Ad.r*Gax* or Ad.h*Gax* at varying MOI and incubated overnight. 10^5 viable cells per well were plated in 6-well plates with inserts containing 8 μm polycarbonate filters, and we measured their migration towards serum-containing media in the lower chamber. Ad.r*Gax* strongly inhibited the migration of HUVECs towards serum, VEGF, bFGF, and TNF-α (Figure 5), as did Ad.hGax (data not shown). Both homologs also inhibited migration of HMEC-1 cells towards bFGF and VEGF (data not shown).

Gax expression inhibits in vivo angiogenesis

Matrigel containing proangiogenic factors, when implanted subcutaneously in mice, can stimulate the ingrowth of blood vessels into the Matrigel plug from the surrounding tissue, and this neovascularization can be estimated by counting CD31-positive cells (36, 50) and/or by measuring hemoglobin concentrations in the plug (35). Moreover, adenoviral vectors diluted in Matrigel implanted as subcutaneous plugs can serve as reservoirs to transduce ECs invading the

plug and drive expression of exogenous genes, producing effects on *in vivo* angiogenesis even when the gene transduced is a transcription factor (55). We took advantage of this observation to test whether exogenously driven *Gax* expression can inhibit angiogenesis *in vivo*, using methodology previously described (36, 50). Matrigel plugs containing bFGF and either Ad.GFP, Ad.*hGax*, or Ad.*rGax* (see Methods) were injected subcutaneously in C57BL/6 mice (N=8 per experimental group). As a positive control for angiogenesis inhibition by a viral vector, we utilized an adenoviral construct expressing a dominant negative form of Akt (Ad.DN-Akt) (22, 50). We observed that the adenoviral vectors expressing *Gax* expression inhibit the neovascularization of the plugs with a potency slightly less than that observed for the Ad.DN-Akt construct (Figure 6), and that the Ad.DN.Akt construct inhibited neovascularization with a potency similar to what has previously been reported (22, 50).

Gax expression downregulates the expression of NF-kB target genes

Next, we compared global gene expression in control HUVECs infected with Ad.GFP with that of HUVECs infected with Ad.r*Gax*. Cells were infected at an MOI=100, incubated 24 hours in normal media, then harvested for total RNA isolation. Global gene expression was compared in two separate experiments using the Affymetrix Human Genome U133A GeneChip® array set (see Methods). In general, the global changes in gene expression induced by *Gax* in this experiment were consistent with an anti-proliferative, antiangiogenic activity. There were 127 probe sets corresponding to known genes showing greater than two-fold upregulation and 115 showing greater than two-fold downregulation. Differences in gene expression between controls and *Gax*-transduced cells ranged from upregulation by approximately 30-fold to downregulation by 238-fold. This pattern was similar in ECs transduced by Ad.h*Gax*, although the magnitude of changes in gene expression tended to be smaller (data not shown).

We first examined genes that were downregulated 24 hours after transduction of HUVECs with Ad.r*Gax* and were immediately struck by the number of CXC chemokines strongly downregulated (Table 1, which shows selected genes that are most strongly downregulated after *Gax* expression and/or most likely to be involved in angiogenesis). Most strongly downregulated of all was GRO-α (CXCL1), a CXC chemokine and a growth factor for melanoma that has also been implicated in promoting angiogenesis (41). Similarly, several other CXC chemokines were also strongly downregulated by *Gax* expression. Many of these peptides are clearly important in mediating EC activation during inflammation and in promoting angiogenesis (18). Consistent with the hypothesis that *Gax* inhibits EC activation, we also observed the downregulation of several cell adhesion molecules known to be upregulated in ECs during activation and angiogenesis, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin (37, 69), all of whose downregulation we have now also confirmed by quantitative real time PCR, Western blot, and flow cytometry (Figure 7). These proteins have all been implicated in leukocyte-EC interactions and are upregulated by pro-inflammatory factors and by VEGF during angiogenesis (37). The pattern of downregulation of these adhesion molecules, coupled with the downregulation of CXC chemokines, suggested to us inhibition of genes normally induced by TNF-α, which in turn suggested the possibility that *Gax* may inhibit nuclear factor κB (NF-κB) activity. Indeed, when we examined our data using GeneMAPP (19) to look for patterns of signal-dependent gene regulation, we found numerous NF-κB-dependent genes (23) downregulated 24 hrs after *Gax* expression (Table 2).

The genes upregulated by *Gax* did not fall into any signal-dependent patterns as striking as the genes downregulated by *Gax* (Table 3). However, we did note results that might suggest

specific pathways upregulated by *Gax*. First, there was a strong upregulation of ALK3 (bone morphogenetic receptor 1a) (34). Although it is known that, in ECs, ALK1 activates ECs through a SMAD1/5 pathway, whereas ALK5 inhibits EC activation through a SMAD2/3 pathway (30), it is not known what role, if any, ALK3 plays in regulating EC phenotype. Second, we noted the upregulation of three CDK inhibitors, $p19^{INK4D}$, $p57^{Kip2}$, and $p21^{WAF1/CIP1}$ (11, 59, 62), suggesting redundant mechanisms by which *Gax* can induce G_1 cell cycle arrest. Finally, we note that *Frizzled-2* was upregulated. Little is known about the potential role of *Frizzled* receptors and Wnt signaling in regulating postnatal angiogenesis, although *Frizzled-2* is known to be expressed in ECs (24) and there is evidence suggesting Wnt signaling inhibits EC proliferation (14).

Gax expression blocks NF-kB binding to its consensus DNA-binding sequence

Given that NF-κB activity has been implicated in the changes in phenotype and gene expression ECs undergo during angiogenesis caused by VEGF, TNF-α, and other factors, and that a number of NF-κB targets have been implicated in inducing angiogenesis (39, 40, 43, 46, 52, 56, 57), we wished to confirm the finding from cDNA microarray studies that *Gax* inhibits NF-κB activity in ECs. We therefore performed EMSAs utilizing nuclear extracts from HUVECs transduced with either Ad.r*Gax* or the control adenoviral vector Ad.GFP to measure binding to a probe containing an NF-κB consensus sequence (61). Specific binding to NF-κB consensus sequence by nuclear extracts from HUVECs transduced with Ad.*Gax* and then induced with TNF-α (10 ng/ml) was much reduced compared to that observed in controls (Figure 8), implying that *Gax* expression interferes with the binding of NF-κB to its consensus sequence.

DISCUSSION

Interactions between tumor and stroma, particularly the ability of tumors to induce angiogenesis, are critical to tumor progression and metastasis (21). At the EC level, the process of angiogenesis involves complex temporally coordinated changes in phenotype and global gene expression in response to alterations in the balance between pro- and anti-angiogenic factors. The stimuli for these changes are communicated from the surface of ECs to the nucleus through multiple overlapping signaling pathways. The peptide factors and the receptors they bind to that activate these pathways have been the subject of intense study over the last decade, because the importance of aberrant EC activation and angiogenesis to the pathogenesis of not just cancer, but of other diverse human diseases, such as atherosclerosis, diabetic retinopathy, psoriasis, and others, has become more apparent (20). Because blocking aberrant angiogenesis has the potential to be an effective strategy to treat or prevent multiple diseases,, understanding how downstream transcription factors integrate upstream signals from pro- and anti-angiogenic factors to alter global gene expression and produce the activated, angiogenic phenotype, has become increasingly important.

Homeobox genes represent a class of transcription factors that, given their ubiquitous roles in controlling body plan formation during embryogenesis, organogenesis, cell proliferation and differentiation, and numerous other important cellular processes (1, 13, 28), would be expected to be involved in regulating the conversion of a quiescent, unactivated EC to the activated, angiogenic phenotype. Indeed, several HOX genes have been implicated in this process. For example, *HOXA9* produces an EC-specific isoforms (*HOXA9EC*), whose expression is downregulated by TNF-α, but whose function is as yet unknown (53). *HOXA9* itself, however, has recently been implicated in inducing migration and tube formation through by upregulating

the *EphB4* receptor (6). Two paralogues, *HOXD3* and *HOXB3*, have been shown to induce an angiogenic phenotype (4, 5, 48), *HOXD3* through its upregulation of integrins $\alpha_V\beta_3$ and $\alpha_5\beta_1$ and urokinase plasminogen activator (4, 5) and *HOXB3* through its inhibition of ephrin A1 ligand expression (48). In contrast, *HOXD10* inhibits the angiogenic phenotype through an as yet undefined mechanism, producing changes in global gene expression consistent with a quiescent, nonangiogenic phenotype (49). Less is known about the involvement of homeobox genes outside the four HOX clusters in regulating angiogenesis. However, at least one such gene, *Hex*, inhibits EC activation and tube formation, and decreases flk-1/KDR expression in ECs (47, 51). These observations are all consistent with an important role for homeobox genes in regulating the transition to the activated, angiogenic phenotype.

Based on our data in this report, we postulate that at least one additional homeobox gene, *Gax*, is also likely to have an important role in regulating EC angiogenesis. We originally isolated *Gax* from a rat aorta library (27), and subsequently we and others found that in the adult its expression is restricted primarily to mesodermal tissues, particularly the cardiovascular system (10, 27, 58). Moreover, *Gax* expression is rapidly downregulated by growth factors and more slowly upregulated by growth arrest signals in VSMCs both *in vitro* and *in vivo* (27, 66, 68), and its expression results in cell cycle arrest (59), p21 induction (59), inhibition of migration (67), and modulation of integrin expression (67). *In vivo*, *Gax* expression in injured vasculature prevents the proliferative response that leads to restenosis after balloon angioplasty (42, 59). Based on these observations, we examined Gax expression in vascular ECs. We found that *Gax* is expressed in this cell type and that it has many of the same activities as in VSMCs. In addition, its expression inhibited EC tube formation on Matrigel *in vivo* (25). These observations led us to the present study, in which we wished to elucidate further the role(s) *Gax* may have in regulating

angiogenesis. Consistent with its regulation in VSMCs, in ECs, *Gax* is rapidly downregulated by serum, proangiogenic, and pro-inflammatory factors (Figures 1 through 4), and is able to inhibit EC migration in vitro (Figure 5) and angiogenesis *in vivo* (Figure 6) These observations led us to examine the mechanism by which *Gax* inhibits EC activation utilizing cDNA microarrays to examine global changes in gene expression due to *Gax*. In addition to observing that Gax upregulates cyclin kinase inhibitors (Table 3) and downregulates a number of proangiogenic factors (Tables 1 and 2), we also found that Gax inhibits the expression of a number of NF-κB target genes (Table 2). Consistent with the cDNA microarray data, Gax inhibits the binding of NF-κB to its consensus sequence (Figure 8).

The NF-κB/Rel proteins are an important class of transcriptional regulators that play a central role in modulating the immune response and promoting inflammation and cancer by regulating the expression of genes involved in cell growth, differentiation, and apoptosis (23). In many cell types, NF-κB promotes cell survival in response to pro-apoptotic stimuli, induces cellular proliferation, or alters cell differentiation (23). The NF-κB/Rel family is composed of at least five mammalian homologs, c-Rel, RelA (p65), RelB, p50/p105 (NF-κB1), and p52/p100 (NF-κB2), which form an array of homo- and heterodimers, known as the NF-κB complex (23). In most cell types, NF-κB exists in the cytoplasm as an inactive form bound to inhibitory proteins known as IκBs. In the classical pathway of NF-κB activation, NF-κB inducers, such as TNF-α and other proinflammatory cytokines, growth factors, UV light, oxidative stress, and bacterial lipopolysaccharide, initiate a signaling cascade ultimately leading to the nuclear translocation of p50/RelA heterodimers, resulting from signal-induced phosphorylation of IκB by IκB kinase (IKK), which targets it for ubiquitination (17, 23). In addition to this classical

pathway of NF-κB activation, a non-canonical pathway involving activation of p52-containing dimmers through regulated processing of the p100 precursor protein (17, 23).

Several lines of evidence have implicated NF-κB activity in regulating EC phenotype during inflammation and angiogenesis and, in particular, the classic activation of RelA-containing heterodimers (39, 40, 43, 52, 56, 57). For example, proangiogenic factors such as VEGF (37), TNF-α (64), and platelet-activating factor (40) can all activate NF-κB signaling and activity in ECs. In addition, inhibition of NF-κB activity inhibits EC tube formation *in vitro* on Matrigel (29, 57), and pharmacologic inhibition of NF-κB activity suppresses retinal neovascularization *in vivo* in mice (70). Moreover, ligation of EC integrin $\alpha_V\beta_3$ by osteopontin protects ECs against apoptosis induced by serum withdrawal (56), an effect that is due to NF-κB-dependent expression of osteoprotogerin (43). Similarly, $\alpha_5\beta_1$-mediated adhesion to fibronectin also activates NF-κB signaling and is important for angiogenesis, and inhibition of NF-κB signaling inhibits bFGF-induced angiogenesis (39). One potential mechanism by which NF-κB signaling may promote angiogenesis is through an autocrine effect, whereby activation of NF-κB induces expression of proangiogenic factors such as VEGF, as has been reported for platelet-activating factor-induced angiogenesis (40). Alternatively, the involvement of NF-κB in activating EC survival pathways is also likely to be important for sustaining angiogenesis (29).

Although NF-κB activity can influence the expression of homeobox genes (7, 53), there have been relatively few reports of functional interactions between homeodomain-containing proteins and NF-κB proteins. The first such interaction reported was between IκBα and HOXB7, where IκBα was found to bind through its ankyrin repeats to the HOXB7 protein and potentiate HOXB7-dependent gene expression (12). More recently, it was reported that IκBα can also potentiate the activity of other homeobox genes, including *Pit-1* and *Pax-8*, through the

sequestration of specific histone deacetylases (65). In contrast, Oct-1 can compete with NF-κB for binding to a specific binding site in the TNF-α promoter (63). In addition, at least one interaction has been described in which a homeobox gene directly inhibits NF-κB-dependent gene expression, an interaction in which Cdx2 blocks activation of the COX-2 promoter by binding p65/RelA (38). It remains to be elucidated if *Gax* inhibits NF-κB-dependent gene expression by a similar mechanism. Regardless of the mechanism, however, this report represents to our knowledge the first description of a homeobox gene that not only inhibits phenotypic changes that occur in ECs in response to proangiogenic factors, but also inhibits NF-kB-dependent gene expression in vascular ECs. These properties suggest *Gax* as a potential target for the antiangiogenic therapy of cancer or other diseases in which aberrantly increased angiogenesis is an important mechanism of pathogenesis. In addition, understanding the actions of *Gax* on downstream target genes, signals that activate or repress *Gax* expression, and how *Gax* regulates NF-κB activity in ECs is likely to lead to a better understanding of the mechanisms of tumor-induced angiogenesis and the identification of new molecular targets for the antiangiogenic therapy of cancer.

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TABLES

Table 1: Genes implicated in angiogenesis that are downregulated by* Gax *expression.

Gene	Fold decrease
CXCL1 (GRO-1/Gro-α)	238.0
Chemokine (C-C motif) ligand 20	237.6
Interleukin-8	181.3
Chemokine (C-X-C motif) ligand 3	119.9
Chemokine (C-X-C motif) ligand 2	79.6
E-selectin	62.6
Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	17.5
Vascular cell adhesion molecule-1	13.0
VEGF-C	5.3
Matrix metalloproteinase 10	4.3
Angiopoietin-1	3.9
Ephrin A1	3.0
Endothelin-1	2.9
Fibroblast growth factor-2 (basic)	2.8
Matrix metalloproteinase 14	2.8
Id3	2.7
Endothelin-1	2.5
Intercellular adhesion molecule-1	2.5
Id1	2.1
Id4	2.1
Endothelial-specific molecule-1 (ESM-1)	2.1

Table 2. Selected NF-kB-influenced genes downregulated by* Gax *expression (*Implicated in angiogenesis)

Gene	Fold decrease
CXCL1 (GRO-1/Gro-α)*	238.0
Interleukin-8*	181.3
Gro-β	97.0
E-selectin*	62.6
Granulocyte chemotactic protein-2	17.5
VCAM-1*	13.0
A20 (TNF-α-induced protein-3)	7.5
TNF-α receptor-associated protein-1 (TRAF-1)*	4.0
Tissue factor*	3.0
Intracellular adhesion molecule-1 (ICAM-1)*	2.5
Endothelin-1	2.5
Heparin-binding EGF	2.1
Endothelial-specific molecule-1 (ESM-1)*	2.1
Interferon regulatory factor-1*	1.7
B94 (TNF-α-induced protein-2)	1.7
Monocyte chemotactic protein-3	1.4

Table 3: Selected genes upregulated by* Gax *expression

Gene	Fold increase
Frizzled homolog 2	30.4
Rab coupling protein	30.1
ALK3 (Bone morphogenetic protein receptor, type Ia)	29.7
Aquaporin 3	19.9
Frizzled	9.8
Pro-alpha I chain of type I collagen	6.4
Thrombomodulin	5.5
Id2	4.6
Integrin β_4 subunit	4.2
β_2-arrestin	2.6
$p19^{INK4D}$	2.5
Insulin-like growth factor binding protein-1	2.5
Cyclin-dependent kinase inhibitor 1C ($p57^{Kip2}$)	2.1
HOXA5	2.1
$p21^{WAF1/CIP1}$	1.5

FIGURE LEGENDS

Figure 1. *Gax* expression is induced in HUVECs by serum and upregulated when serum is withdrawn. Using quantitative real time PCR, *Gax* levels were measured in quiescent HUVECs stimulated with serum and randomly cycling HUVECs placed in low serum medium. *Gax* levels were normalized to β-actin. For this experiment alone, primers for *Gax* and β-actin previously described were used (25). Similar results were obtained with the primer/probe combination described in Methods. **A.** *Gax* is downregulated by serum. **B.** *Gax* is upregulated by serum withdrawal. **C.** PCR gel of the experiment in **A**. Units are arbitrary.

Figure 2. Time course of *Gax* downregulation by mitogens and proinflammatory factors. Quiescent HUVECs were treated with either **(A)** 10% fetal bovine serum (FBS); or 10 ng/ml of either **(B)** $VEGF_{165}$; **(C)** TNF-α; or **(D)** bFGF. At various time points, cells were harvested for extraction of total RNA, which was then subjected to quantitative real time TaqMan RT-PCR with *Gax*- and GAPDH-specific primer/probe sets. (See Methods for sequences and details.) *Gax* mRNA levels were normalized to GAPDH. Units are arbitrary.

Figure 3. *Gax* downregulation by proangiogenic and proinflammatory factors. Quiescent HUVECs were treated with either bFGF, $VEGF_{165}$, or TNF-α, and RNA was harvested for quantitative real time PCR. 10 μg total RNA was subjected to reverse transcription and then quantitative real time PCR using *Gax*- and GAPDH-specific primers. *Gax* message was normalized to GAPDH, and units are arbitrary. (LSM= low serum medium; SFM=serum-free medium; FBS= fetal bovine serum.) **A.** bFGF at various concentrations, cells harvested after 4 hours. **B.** $VEGF_{165}$ at different concentrations, cells harvested after 4 hours. **C.** FBS at various concentrations, cells harvested at 24 hours. **D.** bFGF or $VEGF_{165}$ at various concentrations, cells harvested after 24 hours.

Figure 4. Downregulation of *Gax* expression in endothelial cells by conditioned medium from tumor cell lines. Quiescent HUVECs were treated with either low serum medium (LSM), 10% FBS, or 10% conditioned medium from the indicated breast cancer cell lines. Cells were harvested 4 hours after stimulation, total RNA harvested and real time quantitative RT-PCR performed. *Gax* message level was normalized to GAPDH. Units are arbitrary.

Figure 5. *Gax* inhibits HUVEC migration towards serum. HUVECs were transduced with varying MOI of either Ad.*GFP* or Ad.*rGax* and their migration towards various growth factors and proangiogenic factors determined (see Methods). *Gax* inhibits HUVECs migrating towards **(A)** FBS; and **(B)** FBS, bFGF, $VEGF_{165}$, and TNF-α. Results are expressed relative to control HUVECs not transduced with any virus. Results were analyzed by one-way ANOVA (* indicates $p<0.01$). Similar results were obtained with Ad.*hGax* (data not shown).

Figure 6. Effect of *Gax* expression on angiogenesis in Matrigel plugs. Matrigel plugs (500 ul each) containing 400 ng/ml bFGF and the indicated viral constructs at 10^8 pfu/plug were implanted subcutaneously in the flanks of C57BL6 mice. Plugs were harvested after 14 days incubation for immunohistochemistry using CD31 antibodies and determination of CD31-postitive cells per high powered (400x) field (see Methods for details). Slides were photographed at 200x magnification. (**Legend:** MG = Matrigel plug; ST = stroma surrounding the plug; arrows indicate examples of CD31-positive blood vessels.) **A. No growth factor. B. bFGF alone, no virus. C. Ad.GFP.** Note the infiltration of the plug with CD31-positive vessels such that it is difficult to determine the exact edge of the plug in **B** and **C**. **D. Ad.dN.Akt. E. Ad.h*Gax*. F. Ad.r*Gax*. G. Gross photographs of selected plugs.** Note the hemorrhage into one of the Ad.GFP plugs and the lack of vessels on the capsule of the Ad.*Gax* and Ad.dN.Akt plugs. **H. Vessel counts.** Results are plotted as means ± standard error of the mean, and statistical

differences determined with one-way ANOVA $p<0.0001$ for the overall, and the vessel counts were statistically significantly different from control (Ad.GFP group) for Ad.DN.Akt ($p=0.013$); Ad.h*Gax* ($p=0.008$); and Ad.r*Gax* ($p=0.028$).

Figure 7. Effect of *Gax* expression on the level of E-selectin, VCAM-1, and ICAM-1. A. Quantitative real time PCR. Cells were harvested for total RNA isolation. Total RNA was then subjected to quantitative real time RT-PCR using TaqMan primers and probes specific for each gene and the results normalized to GAPDH. Units were chosen such that controls were set to 100. A very strong downregulation of E-selectin, VCAM-1, and ICAM-1 message level was observed. **B. *Gax* downregulates VCAM-1 and ICAM-1 proteins.** HUVECs were transduced with Ad.*rGax* or Ad.GFP and then incubated overnight. Cells were harvested for total protein and 50 μg protein was subjected to Western blot with appropriate antibodies. (C= control with no virus; GFP=Ad.GFP; *Gax*=Ad.*rGax*). E-selectin could not be visualized in unstimulated HUVECs. **C. *Gax* blocks upregulation of VCAM-1 and E-selectin.** HUVECs were transduced with Ad.*rGax* or Ad.GFP and then incubated overnight, after which they were stimulated with 10 ng/ml TNF-α for one hour. Cells were harvested for total protein and 50 μg protein was subjected to Western blot with appropriate antibodies. Expression of Gax from the adenoviral vector was verified by Western blot with antibodies against Gax previously described (58). **D. *Gax* downregulates cell surface expression of ICAM-1, E-selectin, and ICAM-1**. HUVECs transduced overnight with either Ad.GFP or Ad.*rGax* at an MOI=100 were stimulated with TNF-α 10 ng/ml for 4 hours and then harvested for flow cytometry using appropriate antibodies (see Methods). Ad.r*Gax* blocked the expression of VCAM-1, E-selectin, and ICAM-1.

Figure 8. Gax expression inhibits NF-κB binding to its consensus sequence. A. Gax blocks NF-κB binding to its consensus sequence. HUVECs were infected with adenovirus

containing GFP or r*Gax*, incubated overnight in EGM-2, and then induced with 10 ng/ml TNF-α for 1 hour. Controls were not induced with TNF-α. Nuclear extracts were prepared with the NE-PER nuclear extraction reagent (Pierce). Nuclear extracts were incubated with biotinylated oligonucleotides, containing the consensus NF-κB binding site, and the reactions were electrophoresed on a 6% acrylamide gel. The reactions were transferred to positively charged nylon membrane and detected with the LightShift EMSA kit (Pierce). Arrows denote NF-κB specific bands, and bands at the bottom of the gels represent unbound probe. **B and C. Control EMSAs.** These demonstrate failure of a random sequence oligonucleotide and an NF-κB consensus sequence with a point mutation that abolishes DNA binding to compete with wild-type NF-kB sequence **(B)** and competition with an excess of unlabeled wild-type NF-κB oligonucleotide **(C)**. Legend: NT=no treatment with TNF-α; NV=no virus.

FIGURES

Figure 1

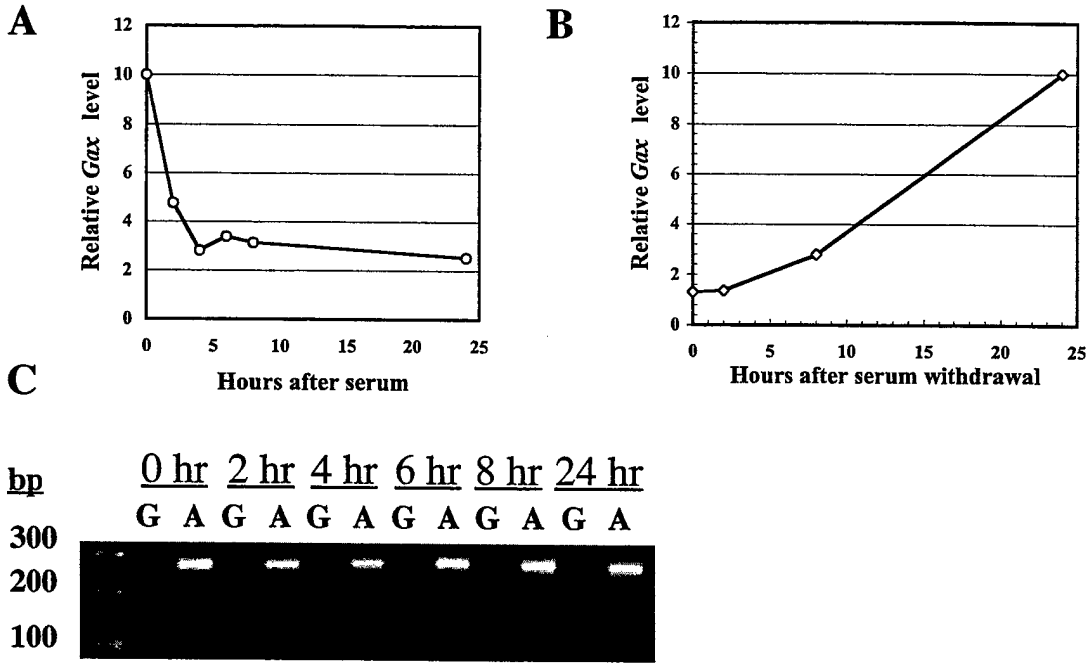


Figure 2

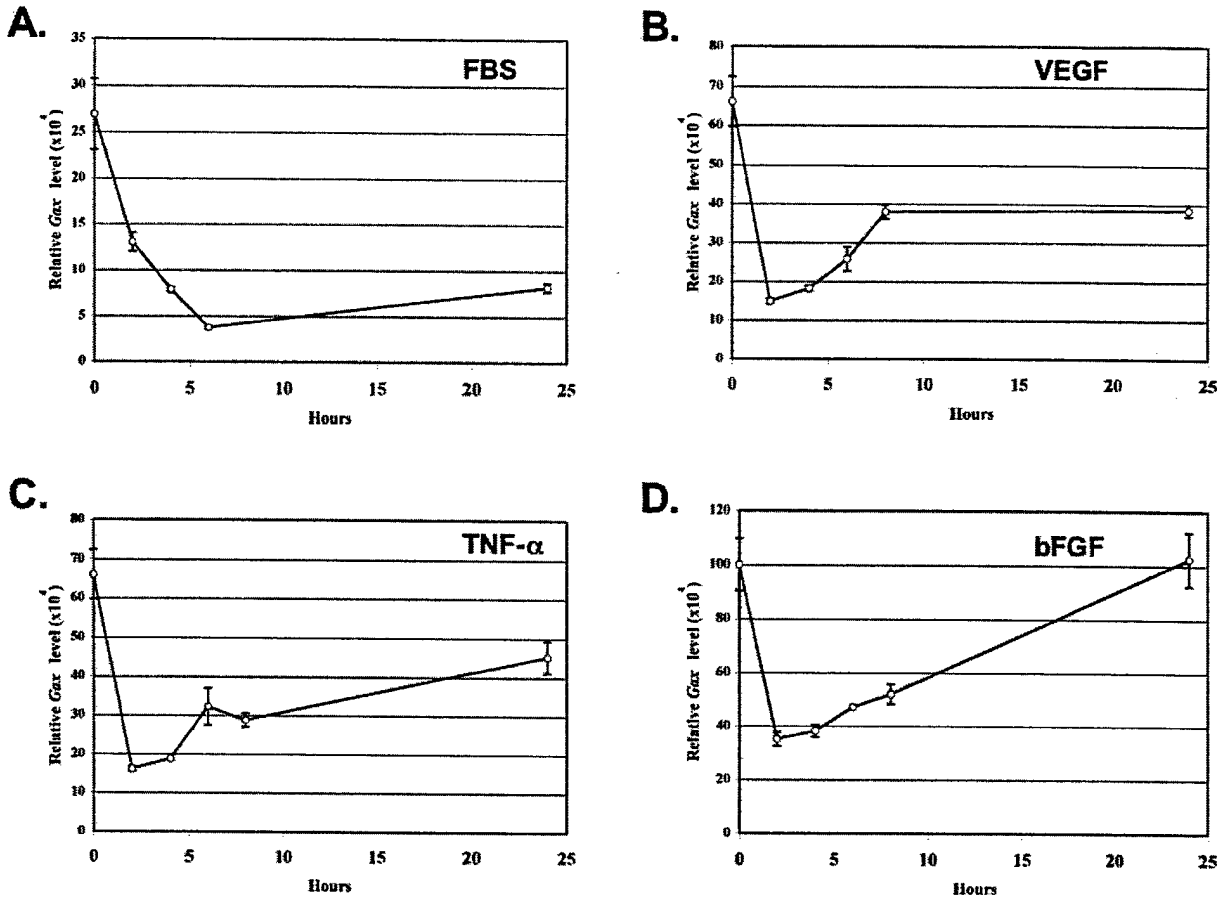

A.
FBS
Relative Gax level (x10⁴)
Hours
B.
VEGF
Relative Gax level (x10⁴)
Hours
C.
TNF-α
Relative Gax level (x10⁴)
Hours
D.
bFGF
Relative Gax level (x10⁴)
Hours

Figure 3

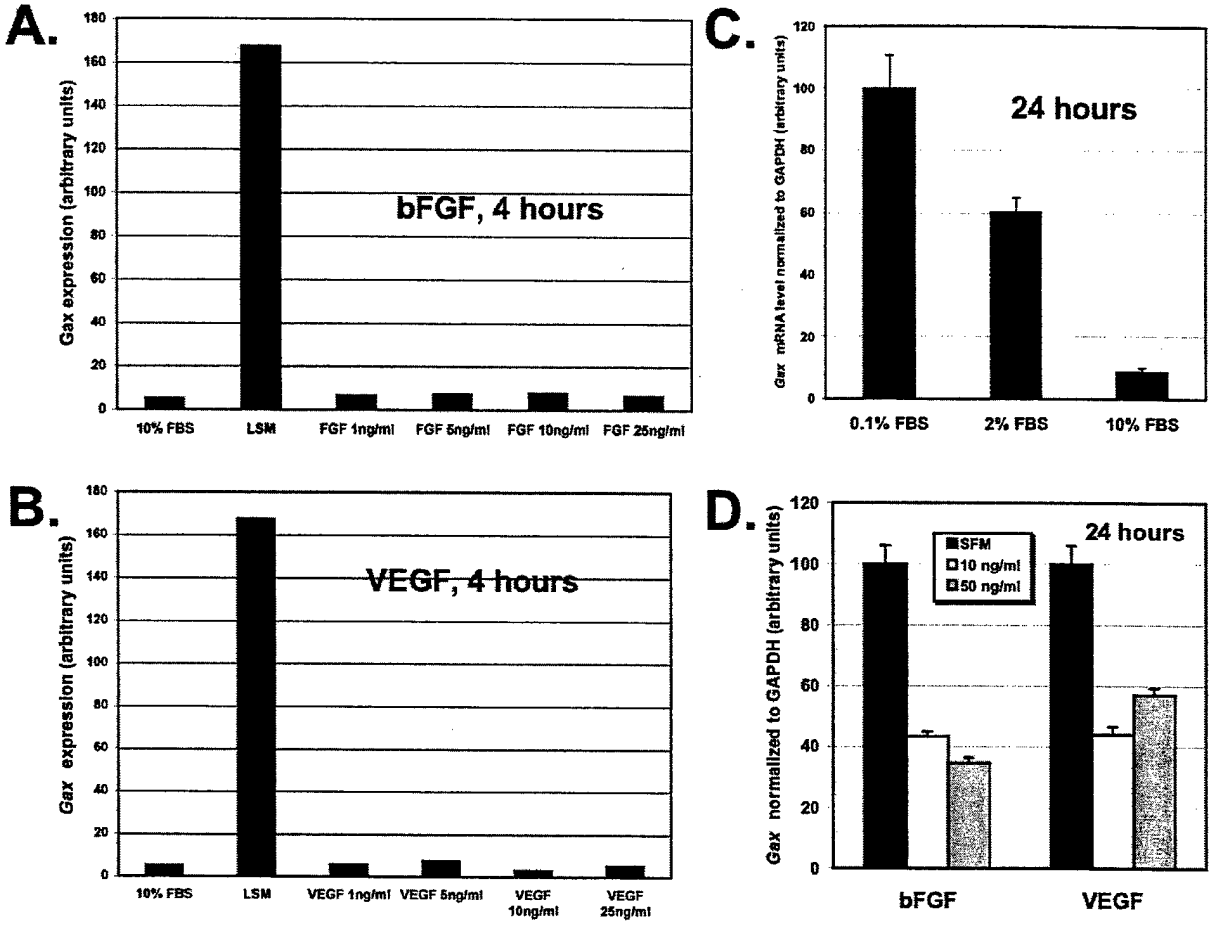

A.
Gax expression (arbitrary units)
bFGF, 4 hours
10% FBS
LSM
FGF 1ng/ml
FGF 5ng/ml
FGF 10ng/ml
FGF 25ng/ml
B.
Gax expression (arbitrary units)
VEGF, 4 hours
10% FBS
LSM
VEGF 1ng/ml
VEGF 5ng/ml
VEGF 10ng/ml
VEGF 25ng/ml
C.
Gax mRNA level normalized to GAPDH (arbitrary units)
24 hours
0.1% FBS
2% FBS
10% FBS
D.
Gax normalized to GAPDH (arbitrary units)
24 hours
SFM
10 ng/ml
50 ng/ml
bFGF
VEGF

Figure 4

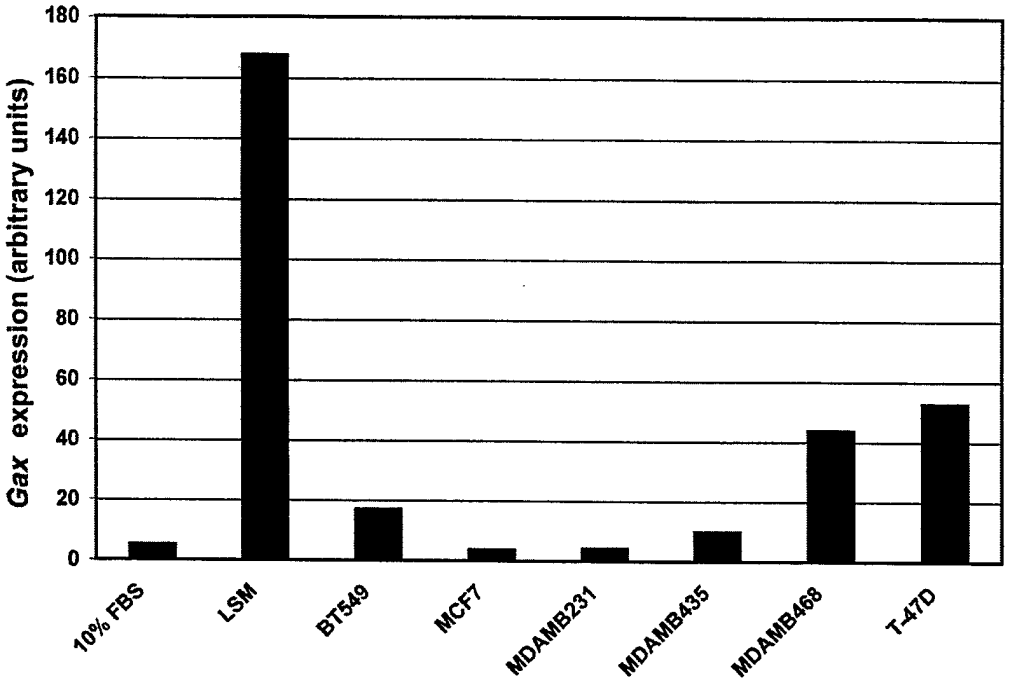

Gax expression (arbitrary units)
180
160
140
120
100
80
60
40
20
0
10% FBS
LSM
BT549
MCF7
MDAMB231
MDAMB435
MDAMB468
T-47D

Figure 5

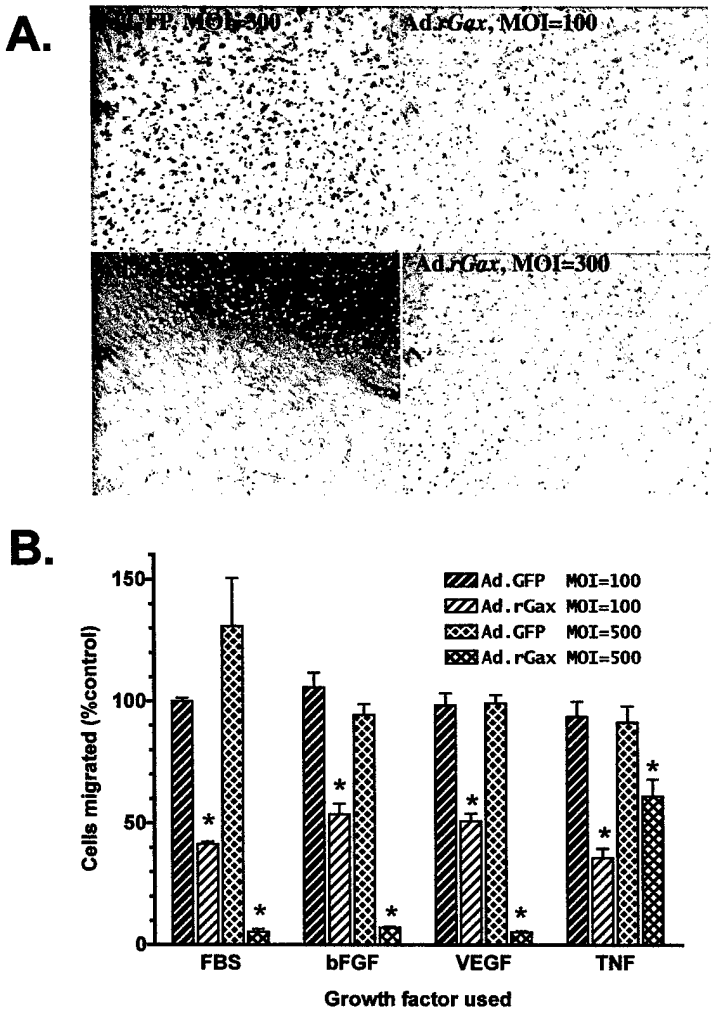
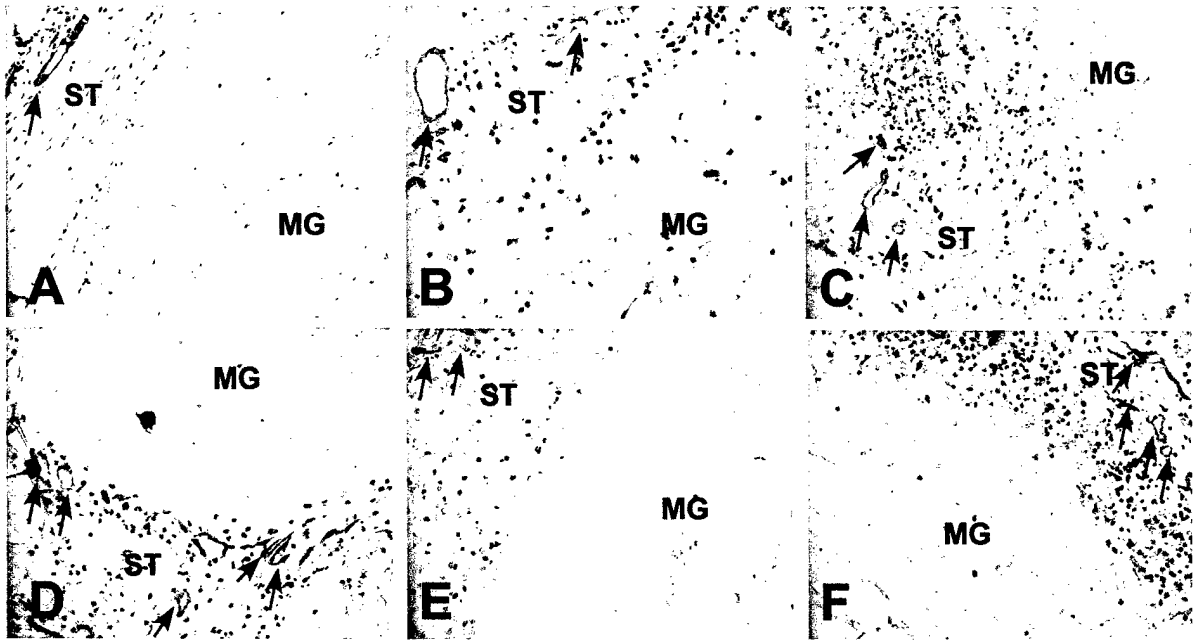
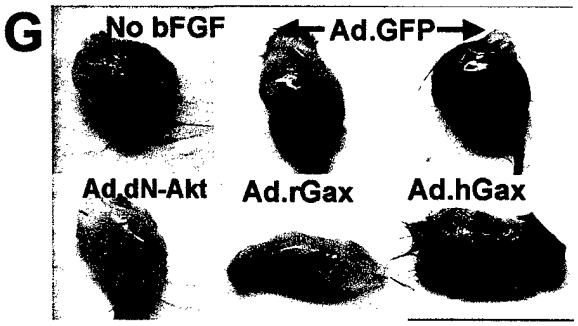

A.
Ad.rGax, MOI=100
Ad.rGax, MOI=300
B.
Ad.GFP MOI=100
Ad.rGax MOI=100
Ad.GFP MOI=500
Ad.rGax MOI=500
Cells migrated (%control)
150
100
50
0
FBS
bFGF
VEGF
TNF
Growth factor used

Figure 6


ST
MG
A
ST
MG
B
MG
ST
C
MG
ST
D
ST
MG
E
ST
MG
F


G
No bFGF
Ad.GFP
Ad.dN-Akt
Ad.rGax
Ad.hGax

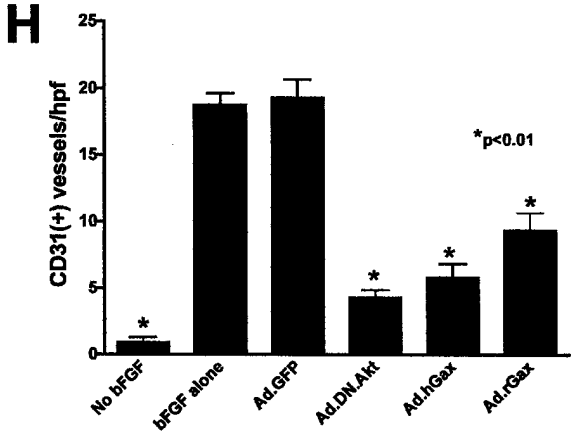

H
CD31(+) vessels/hpf
25
20
15
10
5
0
*p<0.01
No bFGF
bFGF alone
Ad.GFP
Ad.DN.Akt
Ad.hGax
Ad.rGax

Figure 7

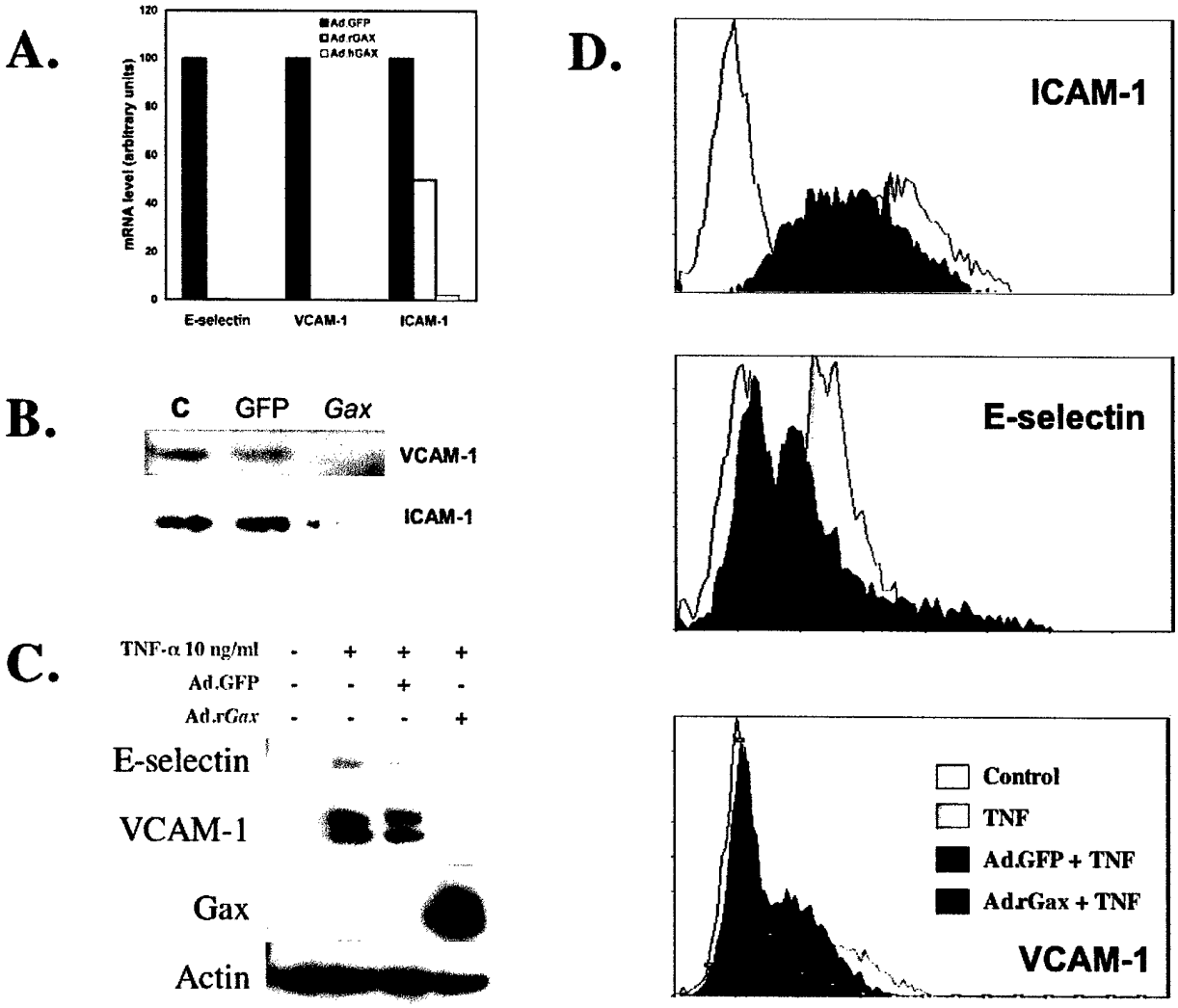
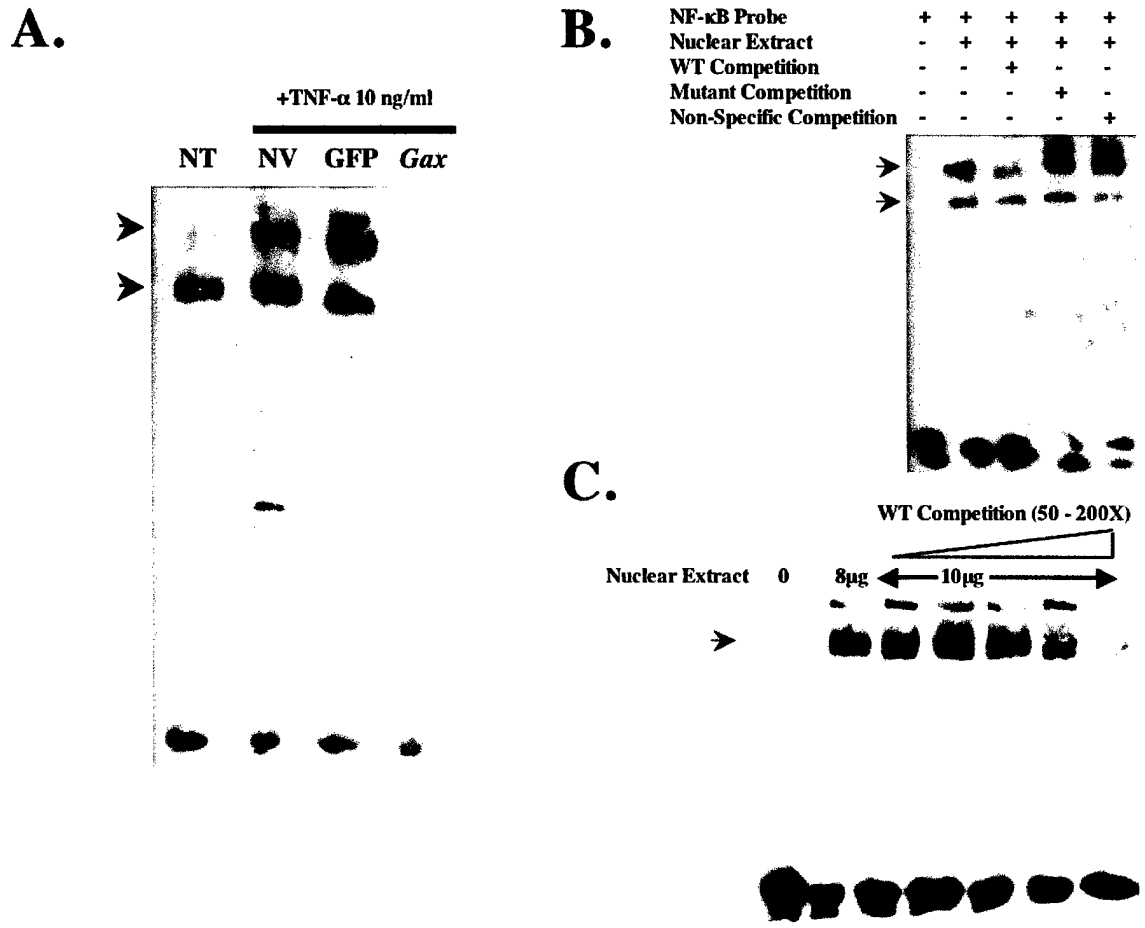

A.
Ad.GFP
Ad.rGAX
Ad.hGAX
mRNA level (arbitrary units)
120
100
80
60
40
20
0
E-selectin
VCAM-1
ICAM-1
B.
C
GFP
Gax
VCAM-1
ICAM-1
C.
TNF-α 10 ng/ml - + + +
Ad.GFP - - + -
Ad.rGax - - - +
E-selectin
VCAM-1
Gax
Actin
D.
ICAM-1
E-selectin
Control
TNF
Ad.GFP + TNF
Ad.rGax + TNF
VCAM-1

Figure 8


A.
+TNF-α 10 ng/ml
NT
NV
GFP
Gax
B.
NF-κB Probe + + + + +
Nuclear Extract - + + + +
WT Competition - - + - -
Mutant Competition - - - + -
Non-Specific Competition - - - - +
C.
WT Competition (50 - 200X)
Nuclear Extract 0 8μg 10μg